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**WASHINGTON UNIVERSITY IN ST. LOUIS**

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Neurosciences

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**SUPRACHIASMATIC NUCLEI DEVELOPMENT:  
A characterization of transcription factors and the influence of retinal innervation  
and VIP signaling**

by

Cassandra Marie VanDunk

A dissertation presented to the  
Graduate School of Arts and Sciences  
of Washington University in  
partial fulfillment of the  
requirements for the degree  
of Doctor of Philosophy

December 2011

Saint Louis, Missouri



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## **ABSTRACT OF THE DISSERTATION**

Suprachiasmatic nuclei development:

A characterization of transcription factors and the influence of retinal innervation and

VIP signaling

by

Cassandra Marie VanDunk

Doctor of Philosophy in Biology and Biomedical Sciences (Neuroscience)

Washington University in Saint Louis, 2011

Professor Paul A. Gray, Chairperson

The suprachiasmatic nuclei (SCN) are highly specialized neural structures with an essential behavioral function; creating the rhythm of the mammalian central clock and entraining that internal clock to the external world. The nuclei each consist of approximately 10,000 neurons, each capable of creating near 24 h rhythms, organized into a highly structured network. While the molecular clockwork underlying the rhythm within neurons and network properties have been well studied, how the nuclei are initially specified and how the network develops is poorly understood. Herein, we seek to elucidate the genes and mechanisms involved in the specification and development of SCN neurons, the SCN network, and circadian function.

We first identified genes expressed relatively discretely with the SCN. Using these genes we provided a detailed analysis of transcription factor (TF) and

developmental-gene expression within the SCN from neurogenesis through to adulthood in mice (*Mus musculus*). Through this analysis we identified a genetically distinct neuroepithelium from which SCN neurons are derived and described a gene cascade through which SCN neurons progress as they become postmitotic. In addition, we observed changes in patterns of TF expression through development indicating maturation of nuclei both prenatally and postnatally. We investigated the contribution of critical circadian components in shaping SCN development by monitoring the localization of TF expression in mouse models that lacked either *Atoh7*, necessary for retinal ganglion cell development, or functional VIP peptide or VIP receptor 2 (*VPAC<sub>2</sub>*, *Vipr2*). We found that maturation of TF expression patterns within the SCN occurred independent of retinal innervation and VIP signaling, suggesting that localizations may reflect intrinsic differences in subsets of neurons within the nuclei rather than induced changes. Finally, we began to define specific TFs necessary for SCN development using a Cre/loxP system to temporally localize TF deletion. We found that the well-conserved TF, *Six3*, is necessary for the initial formation and specification of SCN neurons, but not involved postmitotically in onset or localization of TF or peptide expression. This work begins to reveal aspects of the development of circadian function, by providing a characterization of SCN anatomical development and the first descriptions of TFs necessary for specification.

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# **-CHAPTER 1-**

## **Introduction**

**Suprachiasmatic nuclei as principal circadian rhythm generators**

The mammalian biological system contains several key generators responsible for creating and maintaining rhythms on different time scales for essential body processes including locomotion, respiration, and circadian function. Key characteristics of rhythm generators are that the rhythms are created endogenously without sensory inputs and can be modulated in order for flexibility in our systems. The localization of these centers within the central nervous system and the development and generation of these rhythms have been fundamental points of interest within the field of neuroscience.

Circadian rhythms are endogenous rhythms that occur on a near 24 h cycle. These rhythms are involved in crucial biological processes such as regulating sleep-wake activity and hormone rhythms (Ibuka et al., 1977; Maywood et al., 2007). The principal circadian rhythm generators are the suprachiasmatic nuclei (SCN), a set of bilateral nuclei that act as central pacemakers; creating, maintaining, and translating signals to the rest of the body in near 24 h rhythms (Welsh et al., 1995; Abrahamson and Moore, 2001; Gachon et al., 2004; Morin and Allen, 2006). The SCN reflect a very unique rhythm generating system, as the rhythm is not created solely from the cellular network; rather, each individual cell possesses the machinery to produce an approximate 24 h rhythm and is considered a potential endogenous oscillator (Welsh et al., 1995). These single cells, organized into a highly structured network, communicate with one another to synthesize a single, robust network output that is then transmitted to downstream targets. A crucial aspect of the circadian system is that SCN activity can be modified or entrained by the

external environment. Timing signals (Zeitgebers), such as light, result in the cells of the SCN modifying or shifting the cycle of the rhythm that they are projecting to the rest of the body. In its simplest form then, the circadian system consists of an input (for entrainment to the external environment), the central pacemaker, and an output (for entraining the rest of the body).

The ability of single cells to create a near 24 h rhythm is not unique to the SCN. Other cell types throughout the body also contain the machinery necessary for this rhythm generation (Panda et al., 2002; Storch et al., 2002; Ueda et al., 2002). Thus, there must be additional mechanisms that allow the SCN to be principal pacemakers for the entire mammalian system. What then makes an SCN neuron an SCN neuron? What makes this cell group develop into a functional circadian rhythm generator? The research presented within this thesis seeks to elucidate the genes and mechanisms necessary for the development of the SCN into principal circadian pacemakers.

### **Molecular clock**

Each cell within the SCN has the same oscillatory potential to create a near 24 h rhythm. This central rhythm is produced by set genes, referred to as “clock genes”, which interact in a series of negative and positive feedback loops (for review (Ko and Takahashi, 2006)). These feedback loops include heterodimers of the proteins CLOCK and BMAL1 that enter the nucleus and bind to specific E-box promoter sequences that enhance the transcription of Period (Per) and Cryptochrome (Cry) genes. PER: CRY

heterodimers then translocate back into the nucleus binding upstream sites on Clock and Bmal genes and ultimately inhibiting their own transcription. Additional posttranslational mechanisms such as phosphorylation by casein kinase 1 (Meng et al., 2008) contribute in making this process occur within a 24 h timescale (Reppert and Weaver, 2002). In addition to this main loop, a second loop is thought to aid in the precision and stabilization of the core rhythm by contributing to the regulation of Bmal1 levels (Emery and Reppert, 2004). CLOCK: BMAL1 induce the transcription of nuclear hormone receptors Rev-erb a,b and ROR a,b, which bind response elements (ROREs) of Bmal1 and regulate its transcription, with Rev-erba acting as a repressor (Preitner et al., 2002) and Rora as a trans-activator (Akashi and Takumi, 2005).

As mentioned, the ability to create a 24 h rhythm with this molecular clockwork is not solely a property of SCN neurons (Panda et al., 2002; Storch et al., 2002; Ueda et al., 2002). Not only can cells of other tissue types create a circadian rhythm, they have been shown to also be cell-autonomous oscillators, able to maintain rhythmicity in the absence of the SCN (Yoo et al., 2004). In addition, Yoo and colleagues (2004) demonstrate that different types of peripheral tissues exhibit slightly different circadian properties. These data suggest that although molecular clockwork is present within multiple tissue types that rhythms within each tissue are specialized. However, what underlies these differences in cellular properties between tissue types is just starting to be understood.

**Anatomy and network organization of the SCN**

The SCN are bilaterally located within the ventral anterior hypothalamus just dorsal to the optic chiasm and lateral to the third ventricle. Individually, the SCN contain ~10,000 neurons organized into a highly structured network. Each neuron within the SCN is an endogenous oscillator capable of creating near 24 h rhythms (Welsh et al., 1995). These endogenous oscillators have been shown to vary in their afferent input, peptide expression, and response to stimuli (for review (Moore et al., 2002)). The presence of SCN subtypes has prompted attempts at dividing the SCN anatomically and functionally into discrete zones.

Classically, the SCN is subdivided into two complementary regions based on cells expressing the neuropeptides, arginine vasopressin (AVP) and vasoactive intestinal polypeptide (VIP) (Figure 1). These regions, termed loosely dorsomedial or shell and ventrolateral or core (Moore, 1996), respectively, are divisions more a consequence of convenience for means of comparison across species, than steadfast designations. Although the zones are not concrete, they do provide general distinctions from which network function can begin to be understood.

The SCN core contains the highest density of cells and is characterized by neurons expressing VIP, gastrin releasing peptide (GRP), neurotensin (NT), as well as transient calbindin (Silver et al., 1999; Abrahamson and Moore, 2001; Moore et al., 2002; Karatsoreos et al., 2004; Morin et al., 2006; Kriegsfeld et al., 2008). Both GRP and NT have been shown to play a role in communicating phase resetting signals within the SCN

(Gamble et al., 2007), while VIP acts as a key synchronizing agent between individual cellular oscillators and promotes synchrony (Aton et al., 2005). This region has been shown to receive the highest density of direct photic and non-photic information from the retinohypothalamic tract (RHT) through release of glutamate and PACAP from retinal ganglion cell terminals (Moore and Card, 1985; Cassone et al., 1988; Abrahamson and Moore, 2001). Along with its role in relaying indirect photic signals (Johnson et al., 1989), the inner geniculate leaflet as well as the midbrain raphe nuclei provide non-photic inputs to the SCN core through the release of neuropeptide-Y and serotonin, respectively (Albers and Ferris, 1984; Biello et al., 1994; Meyer-Bernstein and Morin, 1996; Marchant et al., 1997).

Surrounding the central core is the less cell dense SCN shell, marked by neurons expressing AVP and Met-enkephalin (mENK) (Abrahamson and Moore, 2001). Studies have shown rhythmic release of AVP is a key mechanism utilized by the SCN to entrain peripheral clocks (Castel et al., 1990). The SCN shell receives dense projections from the SCN core and modulatory input, in the form of galanin, from other regions within the anterior hypothalamus and preoptic areas (Levine et al., 1994; Abrahamson and Moore, 2001). In addition to the signaling agents mentioned above, electrical (Yamaguchi et al., 2003) and *gamma*-Aminobutyric acid (GABA) signaling mechanisms (Okamura et al., 1990) have also been implicated in both cell-to-cell and core-to-shell communication (Albus et al., 2005).

Investigation in hamsters, mice, and rats demonstrate that the SCN can be distinguished by functionally distinct regions of differential gene expression (Hastings et al., 1999; Hamada et al., 2001; Jobst and Allen, 2002; LeSauter et al., 2003; Karatsoreos et al., 2004). These studies find rhythms in the expression of *Per1*, *Per2*, and *c-fos* mainly limited to the SCN shell, with highest expression levels occurring during subjective day (Sumova et al., 1998; Guido et al., 1999; Hastings et al., 1999; Yan et al., 1999; Schwartz et al., 2000; Dardente et al., 2002; LeSauter et al., 2003; Karatsoreos et al., 2004). Expression of these genes was absent within the core SCN during both subjective light and dark. However, presenting an animal with a light pulse was able to induce rhythmicity of *Per1*, *Per2*, and *c-fos* within this subdivision, a response gated to pulses given during subjective night (Travnickova et al., 1996; Shigeyoshi et al., 1997). Currently, it is unknown if the anatomical and/or functional subdivisions present within the SCN reflect intrinsic genetic differences between groups of cells or if the regions are induced through development.

### **Origins and development of the SCN**

Research focused on development of circadian rhythmicity has centered primarily on when rhythms emerge. However, given that the clockwork is not unique to cells of the SCN, a better way of understanding the development of circadian function would be to ask what is happening within the nuclei before the clock begins ticking. What genes or signals specify SCN neurons? Where are SCN cells derived from? What is necessary for



the network to mature appropriately? One way to begin to understand this complex process of development is to understand what is already known in order to assess what questions remain. Below I summarize what is known about the SCN during three distinct developmental stages. The first developmental time period examines premitotic patterning, during which specific neuroepithelial zones are established from the surrounding germinal matrix and cells begin to undergo mitosis. The second time period, prenatal maturation, encompasses development between the time when cells are postmitotic and the birth of the animal. The third time period, postnatal maturation, reflects a stage that accounts for changes occurring from birth to development of fully functional nuclei. Previous research has utilized rat models; therefore, all ages described refer to the rat gestational timeline unless otherwise stated. Please refer to Figure 2 for a diagrammatic representation of the developmental timeline.

#### **i. Suprachiasmatic neuroepithelium and premitotic patterning**

Tritated thymidine experiments performed by Altman and Bayer (1978a) proposed that the SCN is derived from a special neuroepithelial zone that forms a shallow ventromedian evagination just caudal to the optic recess during the third wave of hypothalamic development. They further described two separate neuroepithelial zones from which the SCN is derived; a dorsal neuroepithelium that gives rise to neurons of the shell, and a ventral zone where neurons of the core are derived. Neurogenesis was described as occurring approximately five days before birth, with neurons of the core

produced first followed by neurons of the shell (Altman and Bayer, 1978b, a), consistent with the spatiotemporal progression seen in mouse (Shimada and Nakamura, 1973; Okamura et al., 1983; Kabrita and Davis, 2008) and hamster (Davis et al., 1990; Antle et al., 2005).

The zone from which the SCN is derived is situated amidst a large array of early morphogens and signaling gradients along the early ventral floor plate. The genes that may be important for early SCN specification are unknown primarily due to the inability to unequivocally mark the neuroepithelium within the broader context of the developing brain beyond anatomical estimates. Within Chapter 2 we aim to find markers of not only SCN neurons but also the neuroepithelium in order to elucidate early SCN development in the context of the developing telencephalon/diencephalon.

## **ii. Prenatal maturation**

By E17 in rat (E15, in mice, (Kabrita and Davis, 2008)), all the neurons of the SCN are postmitotic (Altman and Bayer, 1978a). It is during this time period between the end of neurogenesis and birth that rhythms begin to emerge and transcription factor, neurotransmitter, and peptide phenotypes are evident. Evaluation of gene expression indicates the presence of *Per1* and *Per2* mRNA as early as E17 (Shimomura et al., 2001) closely followed by the expression of *Clock* and *Bmal1* at E19 (Sladek et al., 2004). The first evidence of single cell rhythmicity is in the diurnal expression of *Per1* mRNA present at E17 (Shimomura et al., 2001). Reppert and Schwartz (1984) demonstrated

rhythms in cellular metabolism by observing diurnal fluctuations in the uptake of 2-deoxyglucose occurring as early as E19. By E22, low amplitude, but significant, circadian rhythms in the firing rates of cells are present in SCN neurons (Shibata and Moore, 1987). It is important to note that at this stage, no clock gene proteins can be visualized, suggesting that the fetal molecular clockwork does not drive the circadian fluctuations present. Although distinctions between SCN neurons and rhythms emerge during this postmitotic stage, it remains unclear whether SCN neuronal subtypes are specified during premitotic or postmitotic stages and what is driving fetal rhythms.

### **iii. Postnatal maturation**

Within the first three days of birth the SCN undergoes many functional changes. Retinal fibers begin to innervate the SCN at birth (P0) providing direct light information able to induce expression of *Per1*, *Per2* (Mateju et al., 2009), and *c-fos* (Weaver and Reppert, 1995; McNeill et al., 2011) by postnatal day three (P3). In addition, although low in amplitude, mRNA rhythms in most clock genes are present (Sumova et al., 2006) and endogenous oscillations of *c-fos* in the SCN shell emerge (Bendova et al., 2004). In mature animals a photic light pulse can induce gene expression within the SCN only during subjective night. Developmentally, the gating of cells responsiveness to light is first seen in clock genes by P5 (Mateju et al., 2009) while the induction of *c-fos* is not gated fully until P10 (Bendova et al., 2004; Mateju et al., 2009). Synaptogenesis within the rat SCN was shown to occur slowly until early postnatal stages during which

synapses are rapidly formed until P10, after which the last 30% of synapse connections are made (Moore and Bernstein, 1989). Interestingly, it is not until synaptogenesis is almost complete that the amplitudes of clock gene fluctuations approach adult levels (Sladek et al., 2004), most likely reflecting cell-to-cell communication increasing network synchrony and strength. Additionally, rhythms in Per, Cry, and Clock proteins are present at similar levels to adult by P10 (Ansari et al., 2009).

Onset of photoperiodic entrainment in clock genes develops gradually. Partial entrainment of Per1 and Per2 to photoperiods is detected at P10, but not until P20 for Cry1 and Bmal1 (Kovacikova et al., 2005). This suggests that although components of the clock are present before birth and rhythms can be detected in early postnatal stages, that maturation of the system in the form of innervation, synaptogenesis, afferent, and efferent connections must occur for the system to be complete and fully functional, ie. entrainable, occurring by P20.

### **Influence of the environment on SCN development**

Although circadian rhythms are driven endogenously, it is not fully understood if SCN network organization or functional development is influenced or dependent on outside environmental factors or extra-SCN input. Possible factors could include maternal rhythms, maternal environment, innervation, spontaneous activity, or sensory input.

Circadian rhythms are seen within the fetal SCN even before functional molecular clockwork, suggesting an alternative clock is driving the fetal SCN. Recent research has suggested that the fetal SCN can be thought of as a peripheral oscillator by the mother (for review (Seron-Ferre et al., [IN PRESS])) and strong evidence has shown that maternal signals such as melatonin (Davis and Gorski, 1988; Viswanathan and Davis, 1997) and dopamine (Weaver et al., 1992; Vitaterna et al., 1994; Weaver and Reppert, 1995; Strother et al., 1998) are fully capable of entraining the fetal and even neonatal mouse SCN. Interestingly, pups born to mothers with non-functional circadian rhythms either through SCN lesion (Reppert and Schwartz, 1986; Davis and Gorski, 1988) or genetic modification (Jud and Albrecht, 2006) develop circadian rhythms normally. These reports demonstrate that the fetal SCN and functional circadian rhythms develop independent of the mothers influence.

Light input can influence circadian behaviors. Recently, it has been shown that rearing animals under various photoperiods has a direct consequence on the free-running behavioral period produced (Ohta et al., 2006; Ciarleglio et al., 2011). Ciarleglio and colleagues (2011) demonstrate that not only were the behavioral locomotor periods affected, but that the changes in period length could also be seen in single cell and tissue level rhythmicity. As would be predicted, the disruption or removal of retinal projections to the SCN results in behaviorally free-running animals. These mice are able to maintain a single, although slightly lengthened, rhythmic period (Laemle and Ottenweller, 1998; Wee et al., 2002). Anatomically, removal of the eye by way of physical removal or

through a naturally occurring mutation resulting in anophthalmia has led to changes in both SCN cell number and location (Silver, 1977; Nagai et al., 1992; Tokunaga et al., 1997). In addition, a number of enucleation studies report changes in the number of cells expressing VIP mRNA and/or protein (reduction, (Laemle and Rusa, 1992); increase, (Holtzman et al., 1989; Okamoto et al., 1990; Denis et al., 1993)). The mechanism of how light influences the determination of circadian period and possibly SCN anatomical development is currently unknown. In Chapter 3 we take a closer look at the impact retinal innervation may have on shaping development of the SCN.

### **Transcription factors as tools for understanding development**

Recently, several groups have performed large-scale transcription factor (TF) screens in order to identify genes expressed in relatively discrete populations of cells (Gong et al., 2003; Gray et al., 2004; Lein et al., 2007; Shimogori et al., 2010). The distinct combinations of TFs expressed, at least in part, pattern the differentiation and specification of distinct cell types. Thus, screens of this nature can serve several purposes. First is the identification of cell and nuclei specific genetic markers. Establishing marker genes for populations not only allows for the tracking of specific cells throughout development, but is also essential for the creation of tools for manipulation of the cell type or population of interest. Secondly, identification of genes present at distinct stages can provide useful lineage and developmental information. TF analysis in this manner has propelled the understanding of specification of heterogeneous

cell groups within both the retina (for review (Peters and Cepko, 2002)) and spinal cord (Matisse and Joyner, 1997). As of yet, no direct investigation into TFs present within the SCN or the relationship between TFs and the formation of the SCN and/or circadian rhythmicity has been undertaken.

Overall, the research contained within this thesis aims to identify genes expressed within the SCN during various stages in development in order to create tools for furthering the understanding of the development of SCN subdivisions, the SCN network, and circadian function. In Chapter 2 we seek to find TFs that can relatively discretely identify the SCN from surrounding nuclei during both prenatal and postnatal stages. We then use a subset of the found TFs to characterize the normal development of the SCN from a period before neurogenesis to weaning, when the SCN is mature. In Chapter 3 we utilize the characterized expression patterns to investigate the influence of several crucial aspects of circadian function, namely retinal innervation and VIP/VPAC<sub>2</sub> signaling, on the development of the SCN and formation of mature TF expression patterns. In addition, we begin to elucidate the roles distinct TFs may play in the formation, specification, and development of the SCN (Chapters 2 and 4) by evaluating proper development in mice with either naturally occurring genetic mutations or temporally controlled gene deletions.

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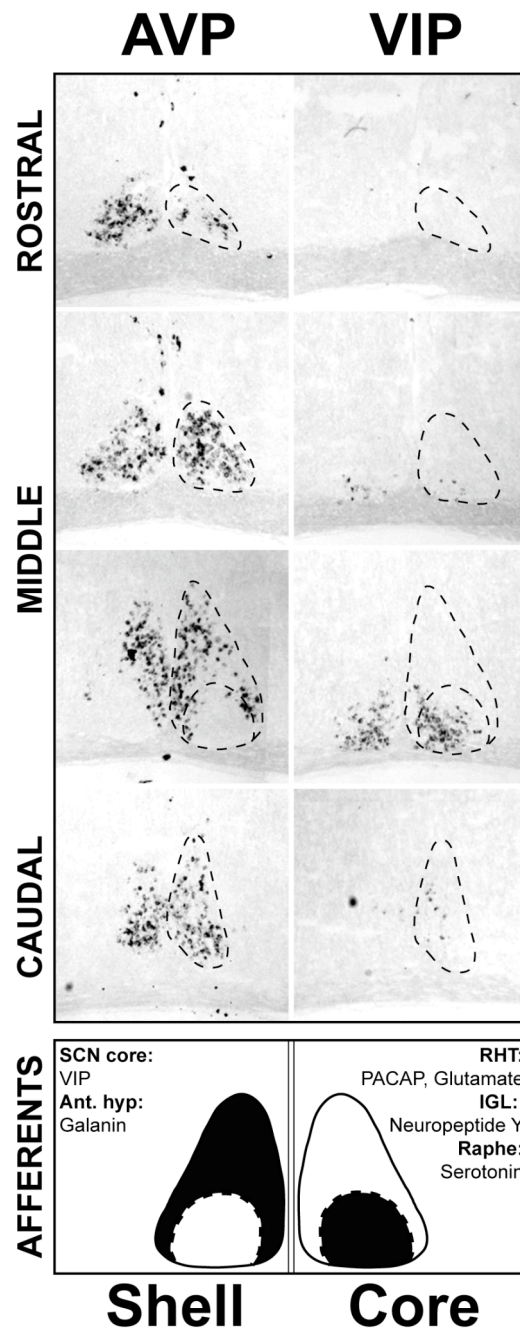
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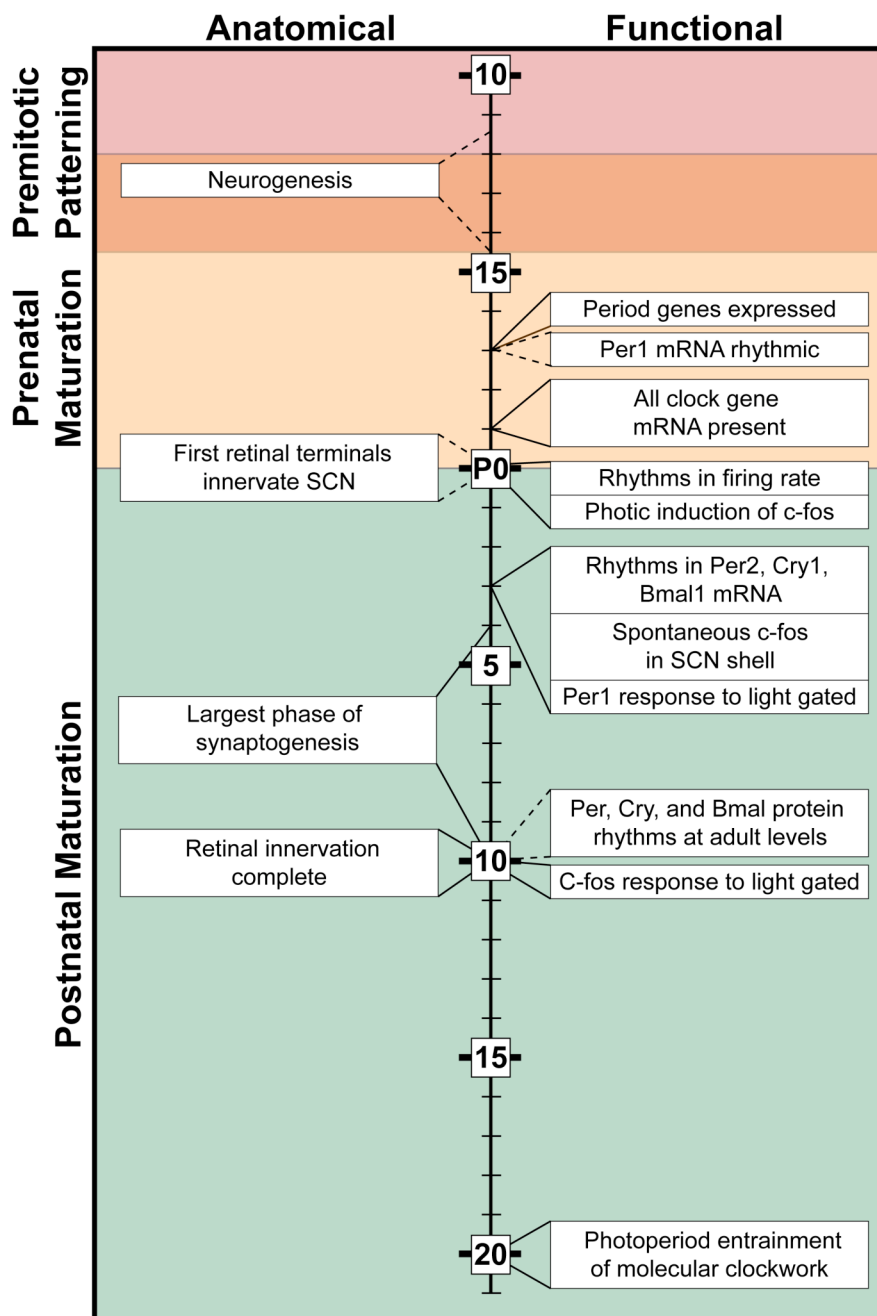
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**Figure 1.** Generalized Suprachiasmatic nuclei organization. Representative *in situ* hybridization images depict expression of arginine vasopressin (AVP) and vasoactive intestinal polypeptide (VIP) mRNA characterizing the SCN shell and core, respectively. Cartoon describes main afferents to each SCN subdivision. (Partially adapted from (Abrahamson and Moore, 2001))



**Figure 2.** Timeline of Suprachiasmatic nuclei development. Diagram depicts anatomical (left) and functional (right) milestones in SCN development displayed along a timeline divided into periods of premitotic patterning, prenatal maturation, and postnatal maturation. Lines from each milestone indicate data obtained using mouse (dashed lines) or rat models (solid lines).

**-CHAPTER 2-**  
**Development, Maturation, and Necessity of Transcription Factors in the**  
**Mouse Suprachiasmatic Nucleus**

This chapter contains the manuscript:

VanDunk C, Hunter LA, Gray PA (2011) Development, maturation, and necessity of transcription factors in the mouse suprachiasmatic nucleus. J Neurosci 31:6457-6467



**Abstract**

The suprachiasmatic nucleus (SCN) of the hypothalamus is the master mammalian circadian clock. The SCN is highly specialized because it is responsible for generating a near 24 h rhythm, integrating external cues, and translating the rhythm throughout the body. Currently, our understanding of the developmental origin and genetic program involved in the proper specification and maturation of the SCN is limited. Herein, we provide a detailed analysis of transcription factor (TF) (Lein et al., 2007) and developmental-gene expression in the SCN from neurogenesis to adulthood in mice (*Mus musculus*). TF expression within the postmitotic SCN was not static but rather showed specific temporal and spatial changes during prenatal and postnatal development. In addition, we found both global and regional patterns of TF expression extending into the adult. We found the SCN is derived from a distinct region of the neuroepithelium expressing a combination of developmental genes: *Six3*, *Six6*, *Fzd5*, and transient *Rx*, allowing us to pinpoint the origin of this region within the broader developing telencephalon/diencephalon. We tested the necessity of two TFs in SCN development, *ROR $\alpha$*  and *Six3*, which were expressed during SCN development, persisted into adulthood, and showed diurnal rhythmicity. Loss of *ROR $\alpha$*  function had no effect on SCN peptide expression or localization. In marked contrast, the conditional deletion of *Six3* from early neural progenitors completely eliminated the formation of the SCN. Our results provide the first description of the involvement of TFs in the specification and maturation of a neural population necessary for circadian behavior.

**Introduction**

The suprachiasmatic nucleus (SCN) of the hypothalamus acts as the master mammalian circadian clock generating a near 24 h rhythm through the cyclic expression of key genes, including crucial transcription factors (TFs). The ability of the SCN to integrate external cues and translate rhythms throughout the body reflects a unique specialization of the nucleus and neurons within (Herzog and Schwartz, 2002). Properties underlying the production of 24 h rhythms have been well characterized; however, the genes involved in specification and development of the SCN are unknown.

The SCN is a heterogeneous group of neurons able to generate unified rhythmic output from individual neuronal oscillators (Webb et al., 2009). Expression of arginine vasopressin (AVP) and vasoactive intestinal peptide (VIP) divide the nucleus into two subdivisions, termed dorsomedial/shell, and ventrolateral/core, respectively. Although these gross divisions do not necessarily reflect differences in cellular properties, these areas have been shown to differ in their projection patterns (Leak and Moore, 2001), innervation (Moga and Moore, 1997; Abrahamson and Moore, 2001), and rhythmicity (Hamada et al., 2001; Yan and Okamura, 2002; Bendova et al., 2004; Karatsoreos et al., 2004).

There is an extensive literature describing the birth dating, onset of neurogenesis, and migration of neurons in the SCN of mammals. Initial experiments in rat estimated the SCN is derived from neuroepithelium caudal to the optic recess during the third wave of hypothalamic development (Altman and Bayer, 1986). In addition, studies in mice have

shown that SCN neurogenesis occurs in a ventrolateral to dorsomedial progression approximately 5 d before birth (Shimada and Nakamura, 1973; Okamura et al., 1983; Kabrita and Davis, 2008) consistent with previous findings in rat and hamster (Altman and Bayer, 1978, 1986; Davis et al., 1990; Antle et al., 2005). Additional information regarding the prenatal SCN remains limited, in part because of the difficulty in identifying SCN boundaries beyond cell density estimates before peptide expression. In addition, no single gene has yet been identified that unambiguously distinguishes all SCN neurons from adjacent neural populations.

Individual TFs can distinguish subsets of cells within heterogeneous populations and provide insight into both development and function (Dasen et al., 2005; Gray, 2008). To date, the study of TFs within the SCN has been either in their relation to clock function or as a consequence of focus on neighboring hypothalamic regions. Investigation of TFs specifically within the SCN may provide insight into SCN development, the presence of genetically distinct areas, and relationships between the SCN and other brain regions.

We hypothesized that SCN function is influenced by TF expression and that these genes play a role in both the formation and maturation of SCN rhythmicity. Thus, we provide a detailed spatiotemporal description of TF and developmental-gene expression within the mouse SCN. We find that the SCN undergoes prenatal and postnatal maturation of gene expression. Furthermore, we find that patterns of TF expression reveal a specific gene cascade from which SCN neurons are derived and can be used to identify

the developing SCN before published markers. In addition, we demonstrate a crucial role for an evolutionarily conserved TF in SCN formation.

**Materials and Methods**

*Animals and housing.* Male and female wildtype CD1 (Charles River), Six3<sup>flx</sup> (Guillermo Oliver, St. Jude Children's Research Hospital, Memphis, TN), ROR $\alpha$ <sup>sg</sup> (002651; The Jackson Laboratory), and Nestin Cre (003771; The Jackson Laboratory) mice were used. Mice were maintained in a normal 12 h light/dark schedule under standard care conditions. Mutant animals were maintained on a C57BL/6 background (Charles River). All experiments were approved by the Animal Studies Committee at Washington University School of Medicine.

*Genotyping.* Mice were genotyped by PCR for disrupted ROR $\alpha$  gene, Six3 floxed, and/or wild-type alleles as previously described (Liu et al., 2006) or as outlined by The Jackson Laboratory. The presence of Cre was determined by the presence or absence of an amplified band using primers (5' GAGTGAACGAACCTGGTCGAAATCAGTGCG 3') and (5' GCATTACCGGTTCGATGCAACGAGTGATGAG 3').

*Tissue acquisition.* Unless otherwise stated, all collections were done at approximately Zeitgeber time 8 (ZT8). Neonatal pups [postnatal day 0 (P0) to P2] or embryos [embryonic day 10.5 (E10.5) to E18.5] from timed pregnant females (morning of plug was E0.5) were anesthetized on ice and either transcardially perfused (E16.5) or immersion fixed in 4% paraformaldehyde (PFA) in 0.1 M PBS, pH 7.4. Older mice were anesthetized with a ketamine/xylazine mixture before perfusion with 4% PFA. All tissues

were postfixed in 4% PFA overnight at 4°C, cryoprotected in 20% sucrose in PBS, frozen in O.C.T. Compound Embedding Medium (Tissue-Tek), and stored at -75°C. Serial sections (14 or 20  $\mu$ m) were cut on a Hacker cryostat and thaw mounted on Superfrost Plus slides (Thermo Fisher Scientific). Five adjacent sets of sections were prepared from each embryonic and postnatal age and stored at 20°C.

*ZT tissue collection.* Tissue was collected as outlined above every 4 h for 24 h from animals (P21-P23) kept in a 12 h light/dark schedule starting at ZT2, two hours after lights on (ZT0). For dark time points, animals were enucleated under red light before perfusion.

*Probe synthesis.* Plasmids for *in situ* hybridization (ISH) probes were acquired from published sources (Gray et al., 2004; Burns et al., 2008; Zhang et al., 2008) or purchased from Open Biosystems. Gene fragments from verified plasmids were linearized by direct amplification by sequence or vector-specific PCR (gene, GenBank accession number, region, forward primer, reverse primer); AVP, BC051997, GTAATACGACTCACTATAGG (T7), AATTAACCCTCACTAAAGGG (T3); BMP7, BC010771, 281-993, GCCTCTGTTCTTGCTGCGCTCC, CCTGGATGGGCAGAGCATCA; Dlx2, BQ443330, 75-797, CGGGCTCCGCTTCACACCTG, ACGGAGTGTCTGGGAAAGTGGA; Hmx2, BC023402, T7, ATTTAGGTGACACTATAG (SP6); Per1, BC039768, 532- 1750,

CCCAGAAAGAACTCATGACTG, GCATCTGGTAAAGCACCAGGG; Ptch1, CB723783, T7, T3; ROR $\alpha$ , BC003757, 273-1054, GCCACCTACTCCTGTCCTCG, GCAGGCTCGCTAGAGGTGGTGT; ROR $\beta$ , BC024842, 331-987, CAGGAACCGTTGCCAACACTGC, CCAGTACGTGGTGGAGTTCGC; Rx, BC024731, 539-1261, CCCGGGATTTCGTCCCGGAGT, TTGGCCTTCAGGCGCAGAGC; SHH, BC063087, 51-998, GAGACCCAACCTCCGATGTG, GAAAGCAGAGAACTCCGTGGC. Digoxigenin (DIG)-labeled antisense and sense RNA probes were made using PCR products as template and T7, T3, or SP6 RNA polymerases (Roche). cRNA probes were purified using Quick Spin columns (Roche) and quantified by spectrophotometry. Probes were used at a concentration of 1-2  $\mu$ g/ml. Sense counterparts of all probes were tested to ensure probe specificity.

*In situ hybridization.* Slides were immersed in 4% PFA, permeabilized with proteinase K, and returned to 4% PFA before being washed in 0.1 M triethanolamine-HCl with 0.25% acetic anhydride. Once blocked in hybridization buffer at 65°C slides were incubated in hybridization buffer containing 1-2  $\mu$ g/ml DIG-labeled antisense cRNA overnight at 65°C. Slides were then washed in 2X SSC at 62°C, washed in 0.2XSSC at 65°C, blocked with 10% normal horse serum (NHS) in 0.1M PBS, and incubated in alkaline phosphatase-labeled anti-DIG antibody (1:2000 in 10% NHS; Roche) overnight. Sections were washed and color was visualized using nitro blue tetrazolium and 5-Bromo-4-

chloro-3-indolyl phosphate (Roche). Staining was stopped after visual inspection. Sections were washed, fixed in 4% PFA, and coverslipped in 90% glycerol, Vectashield Mounting Medium (Vector Laboratories), or UltraCruz Mounting Media with 4', 6' -diamidino-2-phenylindole (DAPI) (Santa Cruz Biotechnology).

*Immunohistochemistry.* Sections on slides were dried at room temperature and rinsed in 0.5% Triton X-100 in PBS (PBS-T) before incubating in 10% NHS in PBS-T. Slides were then placed into primary antibody overnight at 4°C. Primary antibodies used include the following: rabbit (Rb) AVP (1:800; Immunostar), Rb Ki67 (1:500; Thermo Fisher Scientific), goat (Gt) Lhx2 (1:500; Santa Cruz Biotechnology), Gt Lhx9 (1:750; Santa Cruz Biotechnology), mouse TuJ1 (1:1000; Covance), and Rb VIP (1:8000; Immunostar). Primary antibodies were removed, and sections were rinsed in PBS-T before incubation in secondary antibodies for 2 h at room temperature. Secondary antibodies used were either the appropriate Alexa Fluor 488 (1:1000; Invitrogen) or cyanine 3 (1:500; Jackson ImmunoResearch). Sections were washed and coverslipped with Ultra-Cruz Mounting Media with DAPI.

*Image acquisition.* Images were acquired using a Nikon Eclipse 90i microscope, Photometrics Coolsnap HQ2 camera with a Prior Scientific ProScan II motorized translation stage, and acquired in Volocity (PerkinElmer Life and Analytical Sciences) or a Nikon Coolscan V Ed slide scanner with SilverFast SE software (LaserSoft Imaging).



Images were exported as eight-bit JPEG or TIFF files. All images were adjusted for clarity by filtering and/or modifying levels, as necessary, in Photoshop (Adobe Systems).

*mRNA quantification and analysis.* Percentage of SCN area containing either Lhx1 or ROR $\alpha$  expression was calculated by dividing area expressing mRNA by the total estimated SCN area marked with DAPI, in which area was calculated by outlining regions of interest within NIH Image. Whole SCN quantifications of mRNA expression were performed by quantifying intensity of staining relative to background from 10x ISH images using NIH ImageJ. Margins of the SCN in E17.5 to adult animals were determined by cell density marked with DAPI nuclear stain. For statistical analysis of diurnal variation, one-way ANOVA was applied to relative intensity measurements obtained from ZT2, 6, 10, 14, 18, and ZT22 for Six3, ROR $\alpha$ , and Per1. The differences of mRNA relative intensity between ZT6 and ZT18 were analyzed using Student's *t* test.

## **Results**

### **Identification of SCN candidate transcription factors**

The SCN is a specialized neural structure with an essential behavioral function. We hypothesized that aspects of SCN neurons and SCN function are a direct consequence of the TFs they express and that underlie specification. We set out to identify TFs that might be important for both the development and function of the adult SCN by reexamining a published analysis of nearly 1000 TF and TF-related gene expression patterns in mice at E13.5 and P0 (Gray et al., 2004). Two hundred twenty TFs were previously identified as restricted to populations within the hypothalamus of E13.5 and/or P0 mice. From these data as well as other published work (Rivkees et al., 1992; Schaeren-Wiemers et al., 1997; Jean et al., 1999; Wang and Lufkin, 2000) we identified 72 TFs as possibly expressed in discrete patterns of the postnatal (P0; Supplemental Table1) and/or prenatal (E13.5; Supplemental Table 2) anterior hypothalamus that includes the SCN. Of these 72 TFs, 28 appeared potentially expressed within the postnatal SCN itself (Supplemental Table 1). We confirmed the perinatal or adult hypothalamic expression of at least 68% of these 28 TFs using data from the Allen Brain Atlas (Lein et al., 2007).

### **SCN transcription factor expression is diverse and dynamic**

Previous large screens of gene expression within the brain, although extremely informative, have generally lacked the spatial and/or temporal precision to determine the

detailed boundaries of expression within the SCN (Gong et al., 2003; Gray et al., 2004; Lein et al., 2007; Shimogori et al., 2010). To directly address the range of TF expression patterns within the early SCN, we selected a subset of nine postnatal TFs (Lhx1, Lhx2, Lhx9, Otp, Pou2f2 (Oct-2), ROR $\alpha$  (Rora), ROR $\beta$  (Rorb), Six3, and Six6) for detailed ISH or immunohistochemical (IHC) analysis within the P0 mouse SCN. These genes were selected based on their relative levels of expression and specificity within the SCN region and/or previous work, suggesting a possible role in aspects of SCN function (Rivkees et al., 1992; Schaeren-Wiemers et al., 1997; Sato et al., 2004).

We found TFs showed a range of expression patterns suggesting a diverse transcriptional environment (Figure 1). Of the nine postnatal TFs analyzed, six were found specifically expressed in the SCN but not in adjacent hypothalamic nuclei. Compared with a cell density estimate of SCN margins, Six3, Six6, ROR $\alpha$ , and Pou2f2 were expressed throughout the entire SCN, whereas Lhx1 and ROR $\beta$  both displayed regional specificity within the SCN. Conversely, we found two TFs (Lhx2 and Otp) expressed at lateral and dorsal boundaries but not within the SCN. Lhx9 was neither expressed within the SCN nor served as a boundary marker (data not shown).

To determine whether representative patterns of early postnatal TF expression are modified by postnatal maturation, we examined Six3, Lhx1, and ROR $\alpha$  further for expression at P15, just after eyes open. Six3 retained expression throughout the P15 SCN (Figure 2A). Similar to expression at P0, Lhx1 displayed regionalized specificity within the SCN at P15. Expression of Lhx1 covered  $52 \pm 2.6\%$  of the central SCN and partially

overlapped both SCN ventrolateral/core and dorsomedial/shell (Figure 2A). In contrast to expression at P0,  $ROR\alpha$  was no longer present throughout the entire SCN; instead, expression was restricted to only the outer  $71 \pm 2.2\%$  of the SCN, similar in distribution to AVP and  $ROR\beta$ .  $ROR\alpha$  expression did not overlap the VIP-expressing region, seen clearly both in transverse (Figure 2A) and sagittal (Figure 2B) sections through the SCN. We found that expression patterns of *Six3*, *Lhx1*, and  $ROR\alpha$  at P15 were identical to those at P21 and adult (data not shown).

### **Six3 and $ROR\alpha$ show diurnal variation**

The SCN can entrain to photoperiods (Sumova et al., 2006) and can be considered mature by P21. Also, by this time, the amplitude of circadian changes in expression level of clock genes has reached adult levels (Kovacikova et al., 2006). For clock genes, the differences in phase of expression are a consequence of the interactions in the transcription-translation negative feedback loop that underlie circadian rhythmicity. Much of our understanding of the phasic expression of genes within the SCN has relied on quantitative PCR from whole SCN lysates or radioactive ISH, both of which have limited spatial sensitivity (Sunkin, 2006).

Under standard 12 h light/dark conditions, the mRNA levels of central clock genes *Per1*, *Bmal1*, and *Cry1* peak and trough at mid-day (ZT6) and mid-night (ZT18) (Oishi et al., 2000; Shearman et al., 2000), with *Bmal1* and *Cry1* in anti-phase of the mid-day peak of *Per1*. We tested with nonradioactive ISH whether *Per1*, *Lhx1*,  $ROR\alpha$ ,

and *Six3* mRNA displayed variations at these time points. We found that changes in *Six3* and *Per1* were significantly different between mid-day and mid-night ( $p=0.00064$  and  $0.028$  respectively, Student's  $t$  test), while *ROR $\alpha$*  and *Lhx1* levels were not ( $p=0.055$  and  $0.1065$ , respectively).

The spatial localization of *ROR $\alpha$*  at ZT8 in the mature SCN suggested that any change in phase of expression might correspond to changes in spatial localization as well as abundance. We tested the expression of *Six3* and *ROR $\alpha$*  for changes in mRNA intensity and localization of mRNA over a diurnal period ( $n=3$  for each time point). We found *ROR $\alpha$*  displayed temporal variation within the SCN (one-way ANOVA,  $F_{(5,47)}=6.64$ ,  $p=0.0001$ ), with peak at ZT6 and trough at ZT22, in phase with *Per1* (one-way ANOVA,  $F_{(5,21)}=4.56$ ,  $p=0.0089$ ) (Figure 3A, B), consistent with previous PCR-based analyses (Sato et al., 2004). *ROR $\alpha$*  was never expressed within the VIP-expressing region, which indicated a previously unrecognized spatial localization. *Six3* also showed diurnal variations in expression with peak at ZT6 and trough at ZT18 (one-way ANOVA,  $F_{(5,47)}=10.55$ ,  $p<0.0001$ ) (Figure 3A, B).

### **Transcription factors identify the prenatal SCN**

Although the SCN is not functionally mature until postnatal stages, early components of rhythmicity are present in late embryonic life. Some of the earliest rhythms were seen in day/night variations in metabolic activity as early as E19 in the rat (Reppert and Schwartz, 1984) as well as individual rhythmically firing neurons at E22 in

the rat (Shibata and Moore, 1987), which corresponds to approximately E16.5 and E19.5 in mouse, respectively. The expression of *Six3*, *Lhx1*, and *ROR $\alpha$*  in both the early postnatal and mature SCN suggested that these same genes might provide insight into the development of the SCN during the prenatal stage in which cellular rhythmicity emerges.

To clearly identify the perinatal SCN, we determined the onset and localization of AVP and VIP by ISH and IHC. Consistent with previous work in other rodents (Romero and Silver, 1990; Isobe et al., 1995; Ban et al., 1997), we found both AVP and VIP mRNA expression prenatally but protein-labeled cell bodies only at postnatal stages (data not shown). AVP mRNA expression was present in the SCN by E17.5 (two of three animals), while already strongly expressed in the supraoptic nuclei (SON) at this time (data not shown). VIP mRNA expression was present in the SCN by E18.5 (three of five animals) (data not shown). Within the SCN, both peptides were expressed in spatially restricted regions similar to that seen in the adult from their onset, suggesting specification of peptidergic identity occurs before E17.5. The global expression of *Six3* and *ROR $\alpha$*  in the SCN at P0 led us to theorize that their expression would identify the boundaries of the SCN during prenatal maturation. Thus, we analyzed the expression of *Six3*, *Lhx1*, and *ROR $\alpha$*  between the end of neurogenesis at E14.5 and birth (P0).

At E14.5, *Six3* was expressed within a portion of the early hypothalamus in both the presumptive SCN region as well as dorsal areas. In contrast, *Lhx1* was present in discrete bilateral regions distal to the third ventricle within the broader *Six3* domain (Figure 4). A small subset of the ventral most *Lhx1/Six3* region was found to express

ROR $\alpha$  in two of five animals examined, suggesting that the onset of ROR $\alpha$  (Figure 4) occurs at this time point. At E15.5, prior to the onset of VIP or AVP mRNA expression, there was an increase in the area and approximate number of cells expressing Lhx1 and ROR $\alpha$ . Additionally, nuclear staining with DAPI revealed the first signs of aggregation into a cell dense nucleus at this stage (Figure 4, white arrow). However, both Lhx1 and ROR $\alpha$  expression extended beyond the newly forming, cell-dense area, which would be consistent with the presence of neurons that will become the SCN before the onset of clustering. We hypothesized that these genes could be used as prenatal genetic markers to define cells of the SCN more precisely than cell density estimates. By E17.5, ROR $\alpha$  expression encompassed the entire SCN and completely overlapped with Six3, whereas Lhx1 expression was reduced to a central portion of the SCN (Figure 4). Six3 expression, however, also marked cells more dorsal and lateral to the SCN (data not shown). Postnatally, these dorsal populations retained their Six3 expression and comprised the anterior paraventricular nucleus (aPV) and subparaventricular regions of the hypothalamus (data not shown). By P0, Six3 and ROR $\alpha$  expression completely overlapped cell density estimates of SCN margins, whereas Lhx1 continued to remain centralized (Figure 4).

These data suggest that TFs can be used to identify the prenatal SCN before the onset of peptide markers, dense clustering of cells, and onset of rhythmic activity. The data further suggest that the onset of ROR $\alpha$  expression occurs after SCN neurogenesis is complete, consistent with literature describing the delayed onset of ROR $\alpha$  expression

within other brain regions (Nakagawa and O'Leary, 2003). In addition, these data indicate that the SCN is not fully specified by the end of neurogenesis and that SCN maturation occurs in stages both prenatally and postnatally.

### **ROR $\alpha$ does not specify postnatal SCN structure**

In a molecular model of the circadian clock, ROR $\alpha$  positively regulates Bmal1, thereby aiding in the stability of the core rhythm (Sato et al., 2004). Knock-outs of ROR $\alpha$  show slight behavioral abnormalities in circadian rhythms, leading to a shortened circadian period (Sato et al., 2004). To date, it is unknown whether the change in circadian period is attributable solely to the interaction of ROR $\alpha$  with Bmal1 or whether there is an anatomical change within the SCN. Therefore, we investigated the role of ROR $\alpha$  in the development of the SCN by comparing the localization of SCN markers in wild-type mice with *staggerer* mice (ROR $\alpha^{sg/sg}$ ) containing a deletion of the fifth exon in the ROR $\alpha$  gene that causes formation of a premature stop codon and a functionally null protein (Hamilton et al., 1996).

We found the expression of SCN markers in ROR $\alpha^{sg/sg}$  mice were normal. The spatial mRNA expression patterns of AVP and VIP within the SCN as well as that of SCN boundary marker Otp in the hypothalamus (data not shown) remained unaltered in all P15 sg/sg mutants (four of four) compared with wild-type littermates (Figure 5). Thus, although we found that the postnatal expression of ROR $\alpha$  anatomically distinguished the



core of the SCN from its shell, *ROR $\alpha$*  expression was not necessary for the differentiation, presence, or segregation of peptidergic cells within the SCN.

### **Identification of the SCN neuroepithelium**

The ability of *Six3* and *Lhx1* to delineate the prenatal, postmitotic SCN suggested that they might also precisely define the suprachiasmatic neuroepithelium within the early telencephalon/diencephalon boundary zone beyond previous anatomical estimates (Altman and Bayer, 1986). Therefore, we assessed the expression of these genes just before neurogenesis, at E10.5 and during neurogenesis (E11.5 – E13.5) in mice. Specifically, we examined the region from the rostral eye fields extending caudally to the developing pituitary to identify the exact spatial origin of the SCN.

At E10.5, *Six3* marked a broad dorsoventral and rostrocaudal region of the developing floor plate (Figure 6*A* and data not shown), consistent with previous reports (Oliver et al., 1995). At E11.5, *Six3* was restricted to more ventral regions of the early telencephalon/diencephalon but remained expressed within the developing hypothalamus and pituitary (Figure 6*A*, *D*). *Lhx1* expression began at E11.5 in a very small band of cells at the ventral edge of the *Six3*-expressing zone (Figure 6*A*, arrow), allowing for the identification of the early SCN within the telencephalon/diencephalon boundary region (Figure 6*D*). Both a dorsal and rostrocaudal expansion in the number of cells expressing *Lhx1* occurred from E12.5 to E13.5, whereas *Six3* expression became more restricted but still surrounded the base of the third ventricle (Figure 6*A*). The coincident onset of and

increase in *Lhx1* expression during the period of SCN neurogenesis suggests *Lhx1* is one of the first TFs to be expressed in fully postmitotic SCN neurons.

### **Relationship of early SCN to developmental transcription factors**

Identification of the suprachiasmatic neuroepithelium suggested the SCN was derived from a highly discrete region of the developing neural plate. Our initial reanalysis of brain TF expression identified 58 TFs with discrete expression in the area of the E13.5 anterior hypothalamus (Supplemental Table 2). We confirmed the embryonic hypothalamic expression of at least 66% of these 58 TFs using data from Genepaint and Eurexpress expression databases (Visel et al., 2004; Diez-Roux et al., 2011). These TFs included early forebrain genes *Six6* and *Dlx2*. In addition, reduction in the amount of Rx (*Rax*) present in early neuroepithelium has been shown to disrupt SCN location and cell number by an unknown mechanism (Silver, 1977; Tucker et al., 2001). To clarify the origins of the SCN within the developing telencephalon/diencephalon, we compared the expression of *Six3* and *Lhx1* with that of early forebrain genes *Six6*, *Dlx2*, and Rx.

At E10.5, Rx expression overlapped that of *Six3* in the early ventral floor plate (Figure 6B) from the early eye fields through the early developing pituitary (data not shown) consistent with a previous analysis (Bailey et al., 2004). At E11.5, a rostrocaudal gap was evident in Rx expression. Rx persisted within the eye itself and in ventral floor plate just rostral to the developing pituitary (data not shown). *Six6* expression was first seen at E11.5 in a ventrally restricted but overlapping pattern with *Six3* within the entire

rostrocaudal region examined, including within this Rx-negative gap (Figure 6*B, D*). Dlx2 expression was first seen in a small band of ventral cells within the Six3/Six6-expressing and Rx-negative domain (Figure 6*B, D*). Lhx1 was expressed in a subset of the Dlx2-expressing domain (Figure 6*D*).

By E12.5 and E13.5 as Six3/Six6 expression continued, the Dlx2-expressing region expanded rostrocaudally (Figure 6*D*) and dorsally both beneath as well as along the lateral aspects of the third ventricle stratifying the region surrounding the base of the early third ventricle into three subregions (Figure 6*B*). The ventral-most portions of Dlx2 expression overlapped with Lhx1 expression. The mid region retained only Six3/Six6/Dlx2, and the most dorsal aspects lacked Dlx2 (Figure 6*B*). The overlap between Dlx2 and Lhx1 in the most ventral regions suggested that this combination marked newly postmitotic neurons of the SCN, consistent with findings in embryonic rat. The presence of an Lhx1-negative, Dlx2-positive region suggested that Dlx2 might be expressed during the transition period of cells during the last stages of mitosis to early stages of differentiation similar to its expression within the ventral thalamus (Andrews et al., 2003).

The absence of a single TF specific to the SCN suggested that SCN development might also rely on extrinsic morphogens and other signaling peptides to provide key regionalization signals (Burns et al., 2008; Liu et al., 2010). The expression of Six3, Six6, and Dlx2 in the presumptive suprachiasmatic neuroepithelium can be used to identify the immature SCN within the complex early signaling environment of the developing

telencephalon/diencephalon. We found that, at E10.5, the Six3/Rx region also expressed Frizzled5 (Fzd5), a Wnt receptor, in a region surrounding the base of the third ventricle (Figure 6C). This broad dorsal to ventral Six3/Rx/Fzd5 region was further demarcated by the expression of SHH. Six3/Rx/Fzd5 regions lateral to the ventricle expressed SHH, although it remained absent from the most ventral medial region at the base of the third ventricle (Figure 6C). Unlike Rx, Fzd5 expression continued to overlap with Six3/Six6 at E11.5, further defining the SCN proliferative zone in both the dorsoventral (Figure 6C) and rostrocaudal dimensions (Figure 6D). Fzd5 expression at E12.5 and E13.5 was still present beneath the third ventricle but was restricted to the dorsal region overlapping with the Six3/Six6 region only (Figure 6C, D). Fzd5 expression did not overlap with the region containing postmitotic Lhx1 cells and Dlx2, which suggested the expression of Fzd5 likely marked regions of active proliferation. We found that, at E12.5, the most ventral Dlx2- and Lhx1-expressing regions overlapped with TuJ1, a marker of postmitotic neurons, while the more dorsal Dlx2-negative region expressed Ki67, a marker of proliferating cells (data not shown). Throughout this period of neurogenesis, the early SCN was completely devoid of bone morphogenetic protein 7 and lacked the common SHH receptor Patched1 (data not shown).

Our data suggest that the SCN is derived from a proliferative region expressing Six3, Six6, Fzd5, and transient Rx. Furthermore, early postmitotic cells of the SCN express Six3, Six6, Lhx1, and transiently express Dlx2. Figure 7 shows a summary of the relative patterns of developmental-gene expression during SCN development.

### Six3 is required for SCN formation

In the present study, we found that Six3 was continually expressed in the SCN and its progenitors from before neurogenesis to adult when expression was diurnal. This suggested that Six3 has an important role in the SCN and its development. Therefore, we tested whether Six3 is necessary for the formation and maturation of the SCN using a Nestin-cre transgenic to eliminate Six3 ( $Six3^{flox/flox}$ ) (Liu et al., 2006) specifically in progenitors within the neural plate before to specification.

We analyzed the anatomy and expression of Six3 mRNA in  $Six3^{flox/flox}/Nestin-cre^+$  mutants (n=5),  $Six3^{flox/flox}/Nestin-cre^-$  (n=3), and  $Six3^{flox/+}/Nestin-cre^+$  (n=2) littermate controls at E18.5-19.5. We found the Nestin-cre transgenic exhibited a range of Six3 loss in the developing mouse brain, from complete loss to no loss (Figure 8), likely as a result of variation in the onset and duration of cre-recombinase expression.  $Six3^{flox/flox}/Nestin-cre^+$  mice with complete Six3 deletion (n=4 of 5) showed holoprosencephaly and a noticeable enlargement and/or merger of the lateral and third ventricles not seen in littermate controls (data not shown). Furthermore, Six3 ablations lead to loss of the pituitary (Figure 8B). In contrast, retina and optic nerves remained intact (data not shown).

$Six3^{flox/flox}/Nestin-cre^+$  E18.5 and E19.5 animals with complete bilateral loss of Six3 expression showed no expression of ROR $\alpha$  or AVP in the ventral anterior hypothalamus (Figure 8A, asterisks). Importantly, loss of Six3 did not lead to the displacement of the SCN because no ectopic nuclei were present along the ventral surface

either rostral or caudal to the optic chiasm, as assessed through a DAPI nuclear stain (data not shown). Loss of ROR $\alpha$  and AVP was SCN specific because the aPV and SON of *Six3*<sup>flox/flox</sup>/*Nestin-cre*<sup>+</sup> mice retained AVP expression and the dorsal lateral geniculate (dLG) and ventroposterior medial (VPM) nuclei in both E18.5 and E19.5 mutants continued to express ROR $\alpha$  (Figure 8B). In addition, in all mutants tested, the zona incerta (ZI), which normally expresses *Six3*, was unaffected (Figure 8B).

To assess whether absence of SCN at E18.5 and E19.5 was attributable to dissolution of the SCN nucleus after cells became postmitotic or the complete disruption of neurogenesis, we analyzed *Six3*<sup>flox/flox</sup>/*Nestin-cre*<sup>+</sup> mice at E15.5 (n=5) for early postmitotic SCN markers *Lhx1* and ROR $\alpha$  as well as for dense cell clusters. We found that loss of *Six3* leads to absence of both early postmitotic genes, consistent with a disruption in specification. E15.5 *Six3*<sup>flox/flox</sup>/*Nestin-cre*<sup>+</sup> mice displaying partial *Six3* deletion showed a loss of ROR $\alpha$  and *Lhx1* only in regions lacking *Six3* (Figure 8C). *Six3*<sup>flox/flox</sup>/*Nestin-cre*<sup>+</sup> mice that retained expression of *Six3* showed prominent expression of both ROR $\alpha$  and *Lhx1* (Figure 8C). Together, these results show the necessity of a TF for proper SCN formation and development.

## Discussion

Recently, several groups have directly analyzed the expression of large numbers of genes (>1000) within the developing and/or mature hypothalamus by *in situ* hybridization (Gray et al., 2004; Lein et al., 2007; Shimogori et al., 2010) looking for patterns of localization capable of defining small populations of neurons that may correspond to unique functional classes. All of these analyses have shown broad genetic distinctions between hypothalamic nuclei. Other studies have also provided detailed analyses of the roles of several developmental genes in hypothalamic and pituitary ontogeny (Sladek et al., 2004; Davis et al., 2010). However, large anatomical screens, because of their breadth, often lack the spatial detail necessary for a more thorough analysis of specific brain regions. Here we provide a detailed analysis of developmental gene expression in the SCN, describe a specific gene cascade from which SCN neurons are derived, and identify a highly conserved transcription factor necessary for SCN formation.

Altman and Bayer (1986) hypothesized that the early suprachiasmatic neuroepithelium formed from a region just caudal to the optic recess. Our data narrow this description showing that the SCN is derived from a distinct region of neuroepithelium expressing a combination of the developmental genes *Six3*, *Six6*, *Fzd5*, and transient *Rx*, allowing us to pinpoint the suprachiasmatic neuroepithelium within the broader developing telencephalon/diencephalon (for summary, refer to Figure 7 A-C).

ZRDCT anophthalmic mice have decreased expression of Rx protein, and 30% of mutant mice show defects in SCN anatomy and function (Silver, 1977; Tucker et al., 2001). The defects vary widely with respect to disruptions in location and/or cell number, but dense clusters of cells are always seen, indicating formation of nuclei. These data along with our observation of transient Rx before SCN neurogenesis (Figures 6D, 7A, B) suggests that Rx is not necessary for specification of SCN neurons but that loss may disrupt early progenitor domains (Liu et al., 2010).

Altman and Bayer further hypothesized that the suprachiasmatic neuroepithelium was divided into two regions, dorsal and ventral, from which the corresponding dorsomedial/shell and ventrolateral/core were derived. Our data are not consistent with the presence of two genetically distinct regions contained within the suprachiasmatic neuroepithelium. We suggest that the proposed regions outlined by Altman and Bayer are a consequence of temporal proliferation or differential response to non-cell-autonomous cues, such as morphogens or other early-signaling gradients, rather than distinct genetic differences in progenitor type.

We demonstrated that the onset of *Dlx2* and *Lhx1* expression at E11.5 is coincident with the documented onset of SCN neurogenesis (Kabrita and Davis, 2008). This suggests *Dlx2* and *Lhx1* mark neurons as they exit the proliferative zone that will form the SCN. At E12.5/E13.5, the region that gives rise to the SCN contains both proliferating and postmitotic neurons. We demonstrate three zones of overlapping gene expression in the SCN at this age and propose that these distinct TF-expressing zones



represent phases of SCN neuronal development: (I) dorsal Six3/Six6/Fzd5: premitotic/proliferative, (II) mid Six3/Six6/Dlx2: late-mitotic/early-postmitotic, and (III) ventral Six3/Six6/Dlx2/Lhx1: postmitotic (for summary, refer to Figure 7D). Our proposed late-mitotic/early-postmitotic Six3/Six6/Dlx2 zone is similar to the region of migratory “late-forming neurons” outlined by Altman and Bayer (Altman and Bayer, 1986). In addition, our data support the notion of a ventrodorsal gradient in SCN neurogenesis (Kabrita and Davis, 2008) because the expression of Lhx1, a postmitotic marker, was first seen at the most ventral aspects of the early SCN and progressively expanded dorsally as neurogenesis proceeded.

We found that TF expression within the postmitotic SCN was not static but rather showed specific temporal and spatial changes during both prenatal development and postnatal maturation. This was indicated by dynamic expression of Lhx1 prenatally and ROR $\alpha$  postnatally, in which expression of both genes spanned the entire SCN at early stages but became differentially regionalized through development. We propose that changes in localization of gene expression may indicate underlying structural or functional maturation of the SCN. This is supported by the central regionalization of Lhx1 around E17.5, coincident with the perinatal onset of peptide expression. Furthermore, dorsomedial/shell localization of ROR $\alpha$ , evident by P15, is concurrent with the proposed termination of retinal fibers into the SCN (Takatsuji et al., 1995) and onset of photoperiodic entrainment of clock genes (Kovacikova et al., 2005). It would be reasonable to presume that neurons or areas within the SCN may undergo further

specification or specialization as external innervation into the nucleus, such as postnatal retinal innervation (McNeill et al., 2011) and synaptogenesis progress. The identification of TFs with discrete localization within the SCN may reflect additional subdivisions and/or subsets of neurons with distinct functions.

Regional specificity of gene expression, similar to  $ROR\alpha$ , has also been reported for *Period1* and *Period2*, with expression mainly limited to the dorsomedial/ shell during subjective day in rat and hamster (Hamada et al., 2001). Interestingly, some evidence suggests functional division of the SCN into regions of endogenous rhythmicity (dorsomedial/shell) or induced rhythms (ventrolateral/core) (Sumova et al., 1998; Guido et al., 1999; Hamada et al., 2001; Yan and Okamura, 2002; Hamada et al., 2004; Ramanathan et al., 2006). Given the role of  $ROR\alpha$  in stability aspects of the clock through regulation of *Bmal1*, it may be advantageous for the clock to lack stabilizing elements in an inducible region. Furthermore, the pan-SCN expression of *Six3* in phase with *Per1* may suggest a role for *Six3* in maintenance of clock gene expression or underlying circadian rhythmicity.

$ROR\alpha$  is one member of related orphan nuclear receptors that serve not only in the differentiation and development of multiple tissue types, including the cerebellum and thalamus (Becker-Andre et al., 1993; Carlberg et al., 1994; Hamilton et al., 1996; Matysiak-Scholze and Nehls, 1997; Nakagawa et al., 1997; Dussault et al., 1998; Nakagawa et al., 1998; Vogel et al., 2000; Sato et al., 2004), but also in circadian function (Schaeren-Wiemers et al., 1997; Sumi et al., 2002). We found that loss of  $ROR\alpha$

had no effect on either the presence or location of AVP and VIP neurons, indicating that it was not necessary for peptide expression or development of peptidergic segregation.

The role of Six3 in eye development is highly conserved across vertebrates and in *Drosophila* (Simeone et al., 1994; Seo et al., 1999). Six3 has been proposed to play a role in proliferation of retinal precursors (Del Bene et al., 2004), lens formation (Liu et al., 2006), neuroretina specification (Liu et al., 2010), regulation of rhodopsin expression (Manavathi et al., 2007), and early anterior patterning (Kobayashi et al., 2002; Lagutin et al., 2003; Gestri et al., 2005; Lavado et al., 2008). Six3 has been shown widely expressed throughout the anterior brain at early stages, narrowing expression over time and remaining transient for many neuronal populations. We found that Six3 was expressed within the suprachiasmatic neuroepithelium before the onset of neurogenesis into adulthood, suggesting an important role for Six3 in the SCN. Moreover, expression of Six3 in adult mice displayed diurnal variation. Although our data cannot discriminate between whether this variation is a response to rhythmic photic input or reveals an underlying circadian rhythm in Six3 expression, they suggest a link between Six3 and day/night changes.

We demonstrated that Six3 is crucial to the development of the SCN. Using a Nestin-cre transgene to limit loss of Six3 to neural progenitors, we tested the role of Six3 in SCN specification. We showed the loss of Six3 during development leads to elimination of ROR $\alpha$  and AVP expression as well as the absence of dense cell clustering perinatally, consistent with the elimination of the SCN. In contrast, extra-SCN structures,

including hypothalamic SON and aPV, as well as thalamic VPM and Six3 expressing ZI, were still present. This suggests that Six3 may not be acting solely in global early patterning but may have an additional role in specification of the SCN. We propose that the absence of gene expression is a consequence of the failure to form the SCN initially and not a role for Six3 in the maintenance of SCN cell survival, because SCN-specific Lhx1 expression and cell density were also absent at E15.5. These data suggest a necessary role for Six3 in SCN development and that Lhx1 and ROR $\alpha$  represent lineage-specific SCN genes.

Overall, these data demonstrate that TF expression can provide insight into both the specification and development of the SCN. We find that TF expression can describe the early suprachiasmatic neuroepithelium and demonstrate that the SCN undergoes maturation both prenatally and postnatally. Maturation is evident by the changes in localization of gene expression within the SCN over time. It is currently unclear whether the specific loss of gene expression in distinct areas of the SCN is necessary for proper circadian function. However, we find that disruption of at least one TF, Six3, can have large-scale effects on SCN development. Profiling of genes with SCN-specific expression for spatial and temporal onset allows researchers, for the first time, to begin to understand the roles of these genes in the anatomical and functional development of this complex nucleus.

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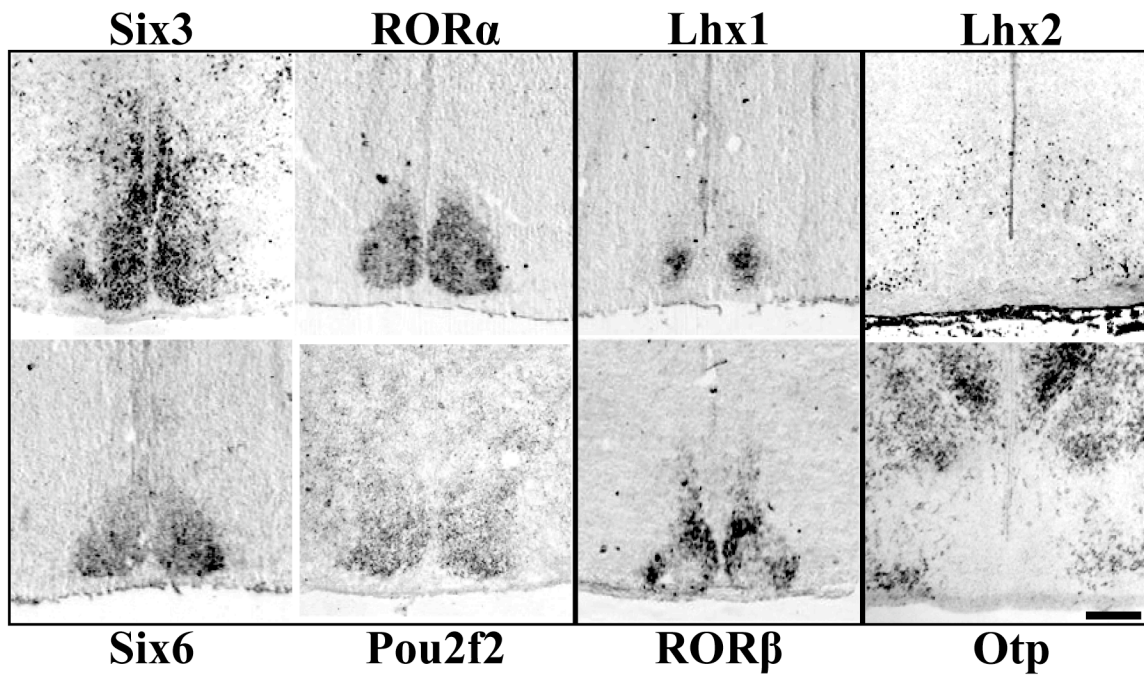
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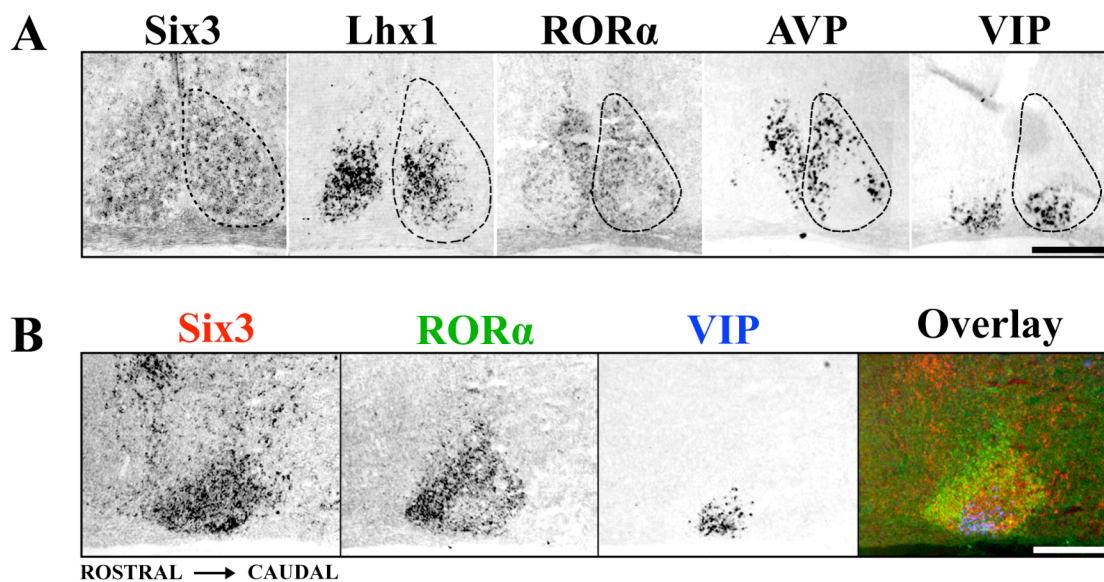
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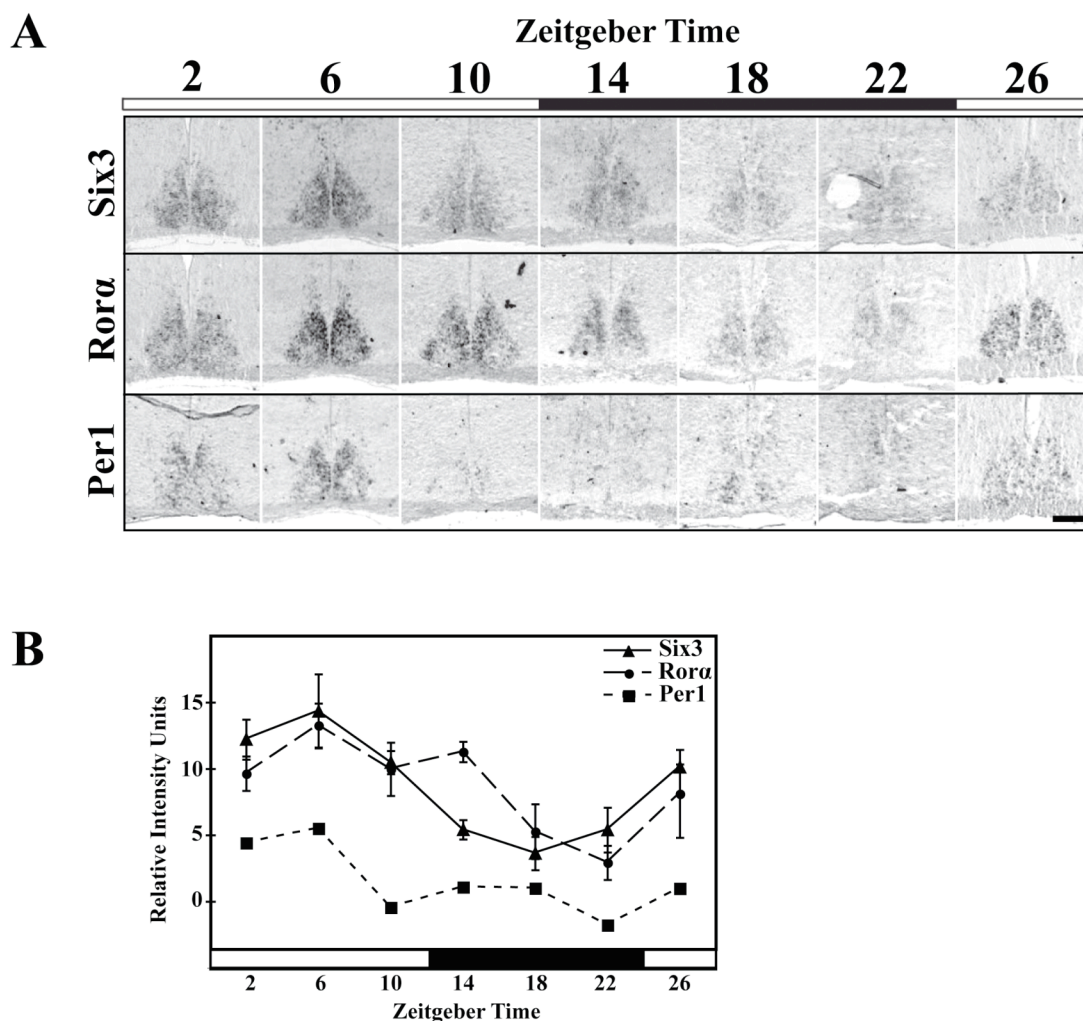




**Figure 1.** Diverse patterns of transcription factor expression in P0 mouse SCN. Representative ISH and IHC images of mid-SCN coronal sections showing specific expression throughout the entire SCN but not adjacent hypothalamus for Six3, ROR $\alpha$ , Six6, and Pou2f2, but restricted localization within the SCN for Lhx1 and ROR $\beta$ . Lhx2 and Otp are expressed outside the SCN, indicating a transcription factor boundary. Scale bar, 250  $\mu$ m.

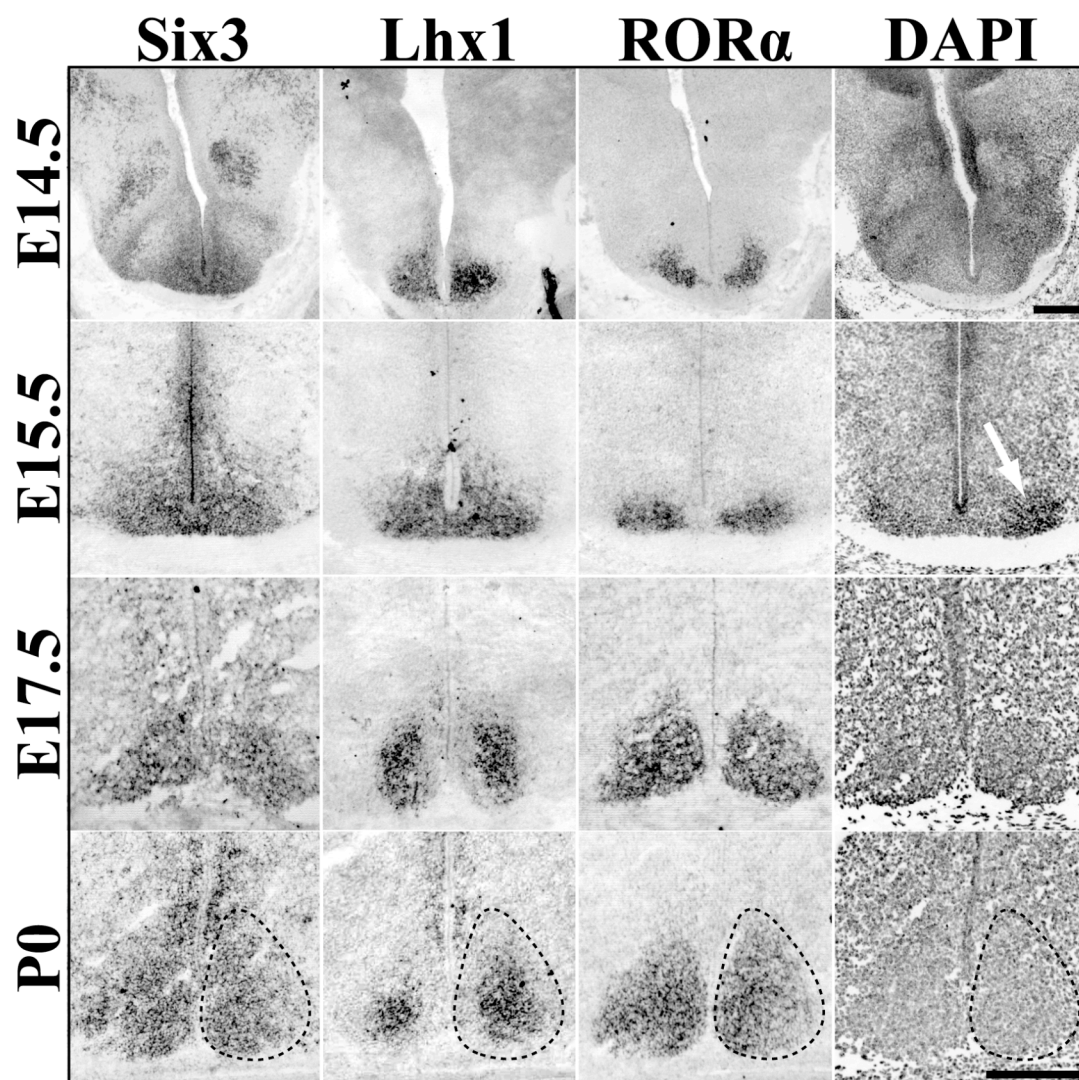


**Figure 2.** The adolescent mouse SCN shows discrete patterns of transcription factor expression. **A**, Representative ISH images showing localization of *Six3*, *Lhx1*, and *RORα* expression within coronal sections of mid-SCN at P15. Note the discrete localization of *RORα* to a region overlapping *AVP* but not *VIP* expression, whereas *Lhx1* overlaps both. Dotted lines indicate the margins of the SCN. **B**, ISH images from adjacent sagittal sections of *Six3* (red), *RORα* (green), and *VIP* (blue) are overlaid in pseudocolor, showing the full rostral to caudal expression of *Six3* throughout the SCN and the localized absence of *RORα* from the *VIP*-expressing region. Scale bars, 250  $\mu$ m.

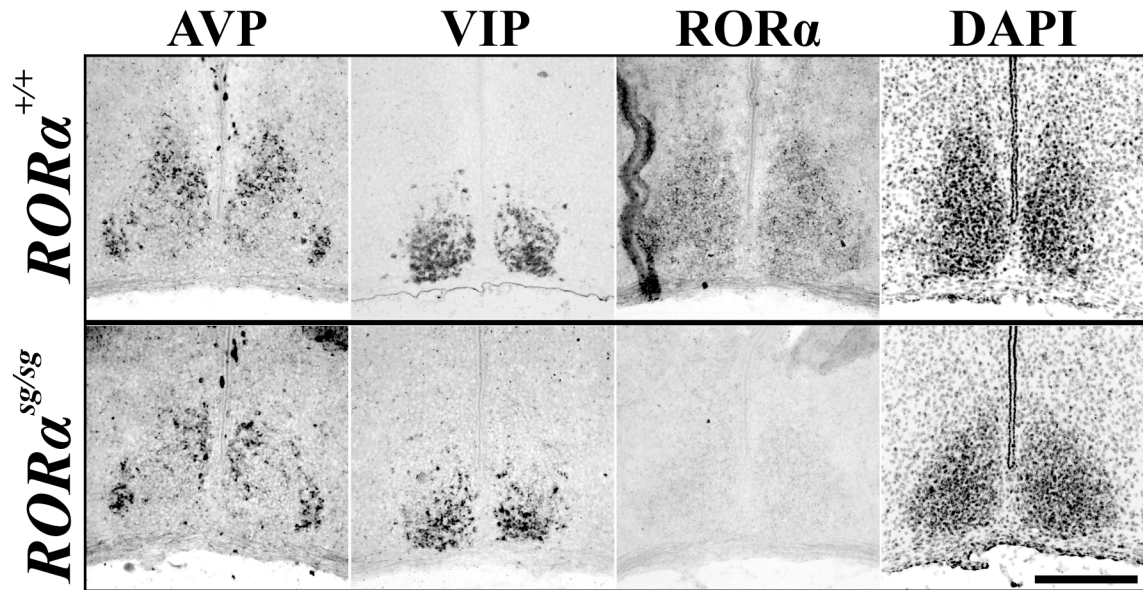


**Figure 3.** Six3 and RORα show diurnal variation in expression. **A**, Representative ISH images showing expression of Six3, RORα, and Per1 mRNA in the coronal mid-SCN in P21–P23 mice raised under a 12 h light/dark cycle and sampled every 4 h (ZT0 indicates lights on). In contrast to Per1 and Six3, RORα is expressed only in the outer shell region. **B**, Quantification of averaged relative mRNA intensity in arbitrary units for Six3, RORα, and Per1 over the same 24 h time period, showing diurnal variation for Six3 (one-way ANOVA,  $F_{(5,47)} = 10.55$ ,  $p < 0.0001$ ), RORα (one-way ANOVA,  $F_{(5,47)} = 6.64$ ,  $p = 0.0001$ ), and Per1 (one-way ANOVA,  $F_{(5,21)} = 4.56$ ,  $p = 0.0089$ ), with peak at ZT6 and trough at ZT22 for RORα and Per1 and ZT18 for Six3 ( $n = 3$ ). Black bars indicate 12 h of dark. Error bars show SEM. Scale bar, 250  $\mu$ m.





**Figure 4.** Dynamic transcription factor expression during SCN prenatal maturation. Representative ISH images of *Six3*, *Lhx1*, and *RORα* mRNA expression in the embryonic mouse SCN (E14.5 to P0), showing spatial specificity and prenatal maturation of transcription factor expression. Dotted lines indicate margins of SCN estimated from the cell density marker DAPI (example shown in rightmost panels). White arrow at E15.5 indicates onset of visible clustering of cells. Note change in *Lhx1* pattern from global to centrally restricted between E15.5 and P0. E17.5 *Lhx1* image was obtained from tissue cut at a different angle and thus the appearance of larger SCN compared with *Six3* and *RORα*. Scale bar, 250  $\mu\text{m}$ .



**Figure 5.**  $ROR\alpha$  is not necessary for normal SCN peptide localization. Representative ISH images of AVP and VIP mRNA expression, markers of SCN subdivisions, as well as transcription factor  $ROR\alpha$  and inverted DAPI nuclear stain in P15  $ROR\alpha$  mutant ( $sg/sg$ , bottom) and wild-type littermate ( $+/+$ , top) mice. Note that segregation of peptidergic expression patterns was similar to littermate control. Scale bar, 250  $\mu$ m.

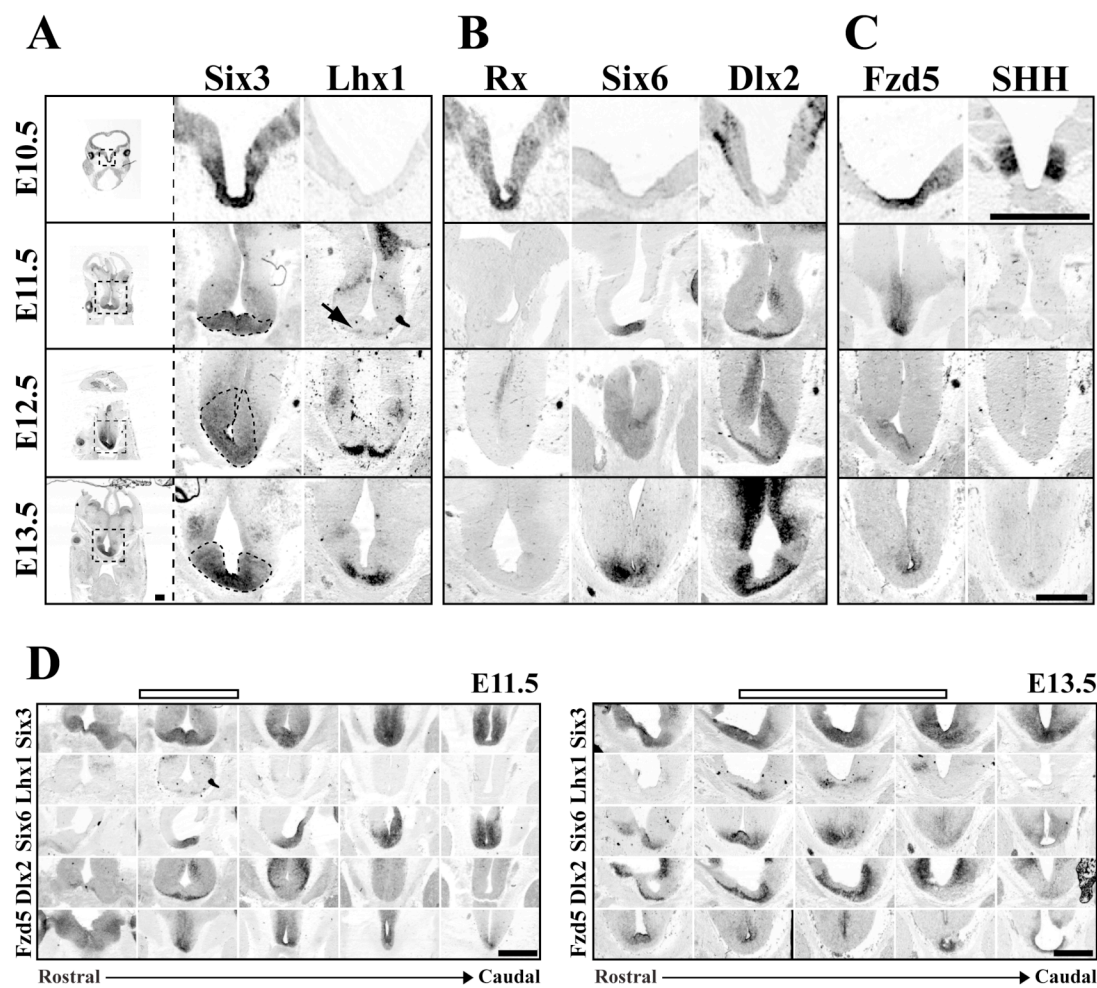


FIGURE 6

**Figure 6.** SCN is derived from a distinct progenitor domain. Representative images of Six3 and Lhx1 (**A**), Rx, Six6, and Dlx2 (**B**), and Fzd5 and SHH (**C**) mRNA expression (columns) in SCN progenitor domain from E10.5 to E13.5 (rows). Leftmost column shows entire coronal section, with dotted box indicating approximate region of enlarged images in corresponding row to the right. Dotted black lines in enlarged Six3 images indicate hypothesized early SCN margins outlined by Six3 expression from E11.5 to E13.5. Note at E10.5 that the region that will give rise to SCN neurons expresses a combination of Six3, Rx, and Fzd5. By E11.5, Rx expression is lost and Six6 expression begins. Also note the E11.5 onset of Lhx1 (black arrow) and Dlx2 on the most ventral aspects of tissue beneath the third ventricle. Scale bar, 500  $\mu$ m. **D**, Rostrocaudal location of SCN progenitor domain. ISH images depict patterns of Six3, Lhx1, Six6, Dlx2, and Fzd5 mRNA expression (rows) in transverse plane from rostral eye fields to caudal developing pituitary at E11.5 and E13.5. Open bars above indicate the developing SCN within the rostrocaudal axis. Note that, although expression of Six3 and Six6 is consistent along the ventral surface from rostral eye fields to the early pituitary, Lhx1 and Dlx2 mark discrete zones within this region at both E11.5 and E13.5. Please note in **D** that E11.5 depicts same transverse images as shown in **A–C**, and E13.5 shows the same Six6 and Fzd5 images shown in **A–C** now within the rostrocaudal context of the developing brain. Scale bar, 500  $\mu$ m.



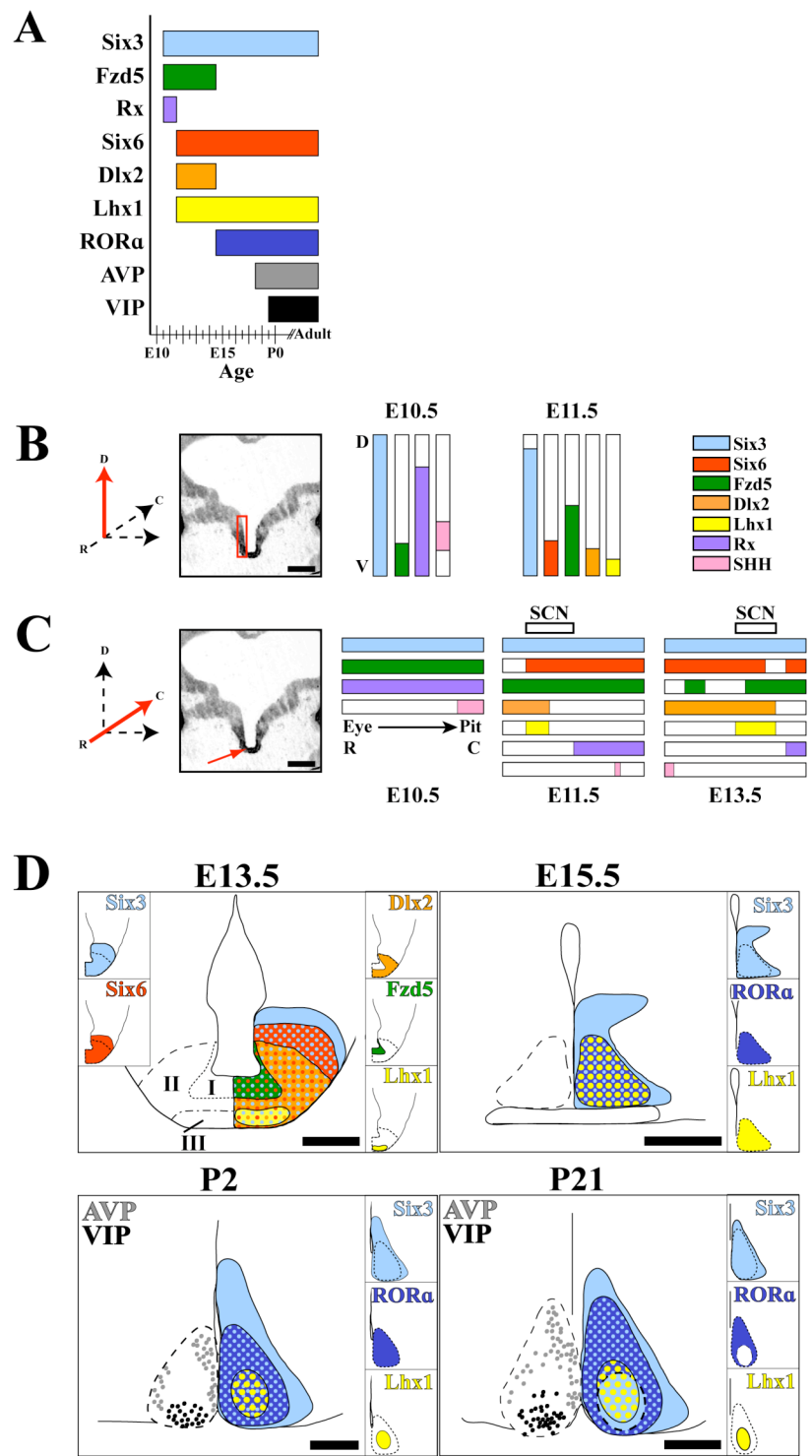


FIGURE 7



**Figure 7.** Summary of SCN development. **A**, Bar graph indicates onset and duration of transcription factor and peptide mRNA expression from E10.5 to adult in the mouse SCN. **B**, **C**, Diagrams summarize dorsoventral and rostrocaudal extent of developmental gene expression within the early telencephalon/diencephalon containing the hypothesized early SCN. Red arrows on orientation axes show directionality of diagram in relation to indicated red regions. **B**, Summary of dorsoventral (D–V) extent of mRNA expression (colored bars) within the same region of anterior hypothalamus, indicated by red box in image showing *Six3* expression, at E10.5 (left) and E11.5 (middle). Scale bar, 250  $\mu$ m. Colors correspond to genes in legend (right). Note the absence of *SHH* but presence of *Fzd5* within the ventral most domains, i.e., putative early SCN. **C**, Summary of rostrocaudal (R–C) extent of mRNA expression within the midline (red arrow) of the ventral developing telencephalon/diencephalon at E10.5 (left), E11.5 (middle), and E13.5 (right). Scale bar, 250  $\mu$ m. The most rostral point is marked by the retina (Eye) and caudal by the developing pituitary (Pit). Colors correspond to genes in legend in **B**. Open top bars at E11.5 and E13.5 indicate rostrocaudal extent of putative SCN progenitor domain within larger developing brain. **D**, Diagrams of SCN transcription factor localization indicate expression during neurogenesis (E13.5), within the prenatal postmitotic (E15.5), early postnatal (P2), and functionally mature SCN (P21). Dotted lines on left indicate the margins of the SCN. Insets indicate expression pattern of individual transcription factors. Colored dots indicate regions of coexpression. Note hypothesized zones of developing SCN at E13.5: I, premitotic/proliferative; II, late-mitotic/early-postmitotic; III, postmitotic. Also note the changes in localization of both *Lhx1* and *ROR $\alpha$*  over time. Scale bars, 250  $\mu$ m.

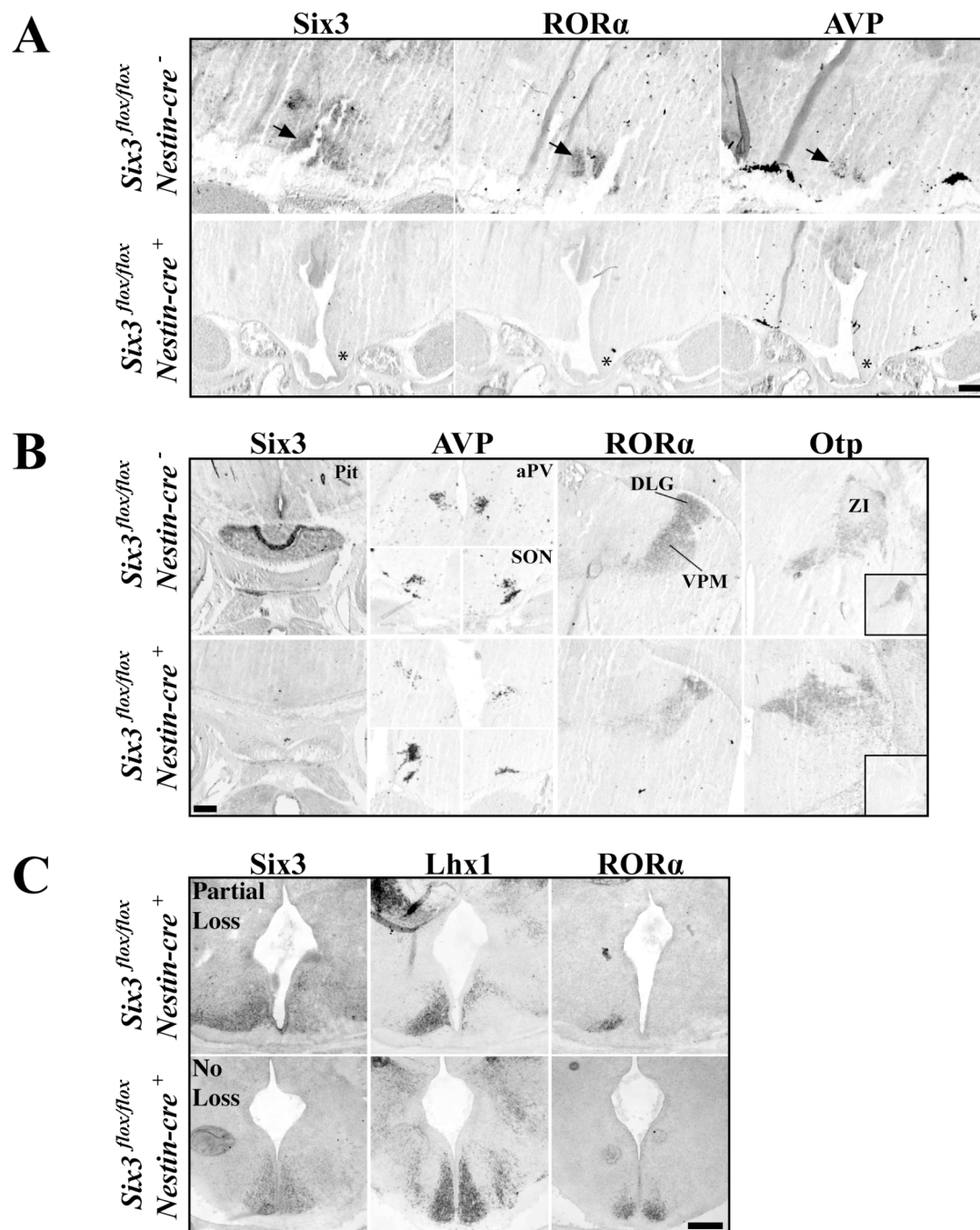


FIGURE 8

**Figure 8.** Six3 is necessary for SCN formation. Expression of Six3, ROR $\alpha$ , AVP, and Otp in *Six3<sup>flox/flox</sup>/Nestin-cre<sup>+</sup>* compared with *Six3<sup>flox/flox</sup>/Nestin-cre<sup>-</sup>* littermate controls. **A**, Expression of Six3, ROR $\alpha$ , and AVP in SCN (arrows in top) are completely eliminated in conditional Six3 knock-outs, shown at E18.5 (asterisks). **B**, Marker analysis of the pituitary, hypothalamic (aPV and SON), and thalamic (dLG, VPM, and ZI) nuclei show that complete disruption of Six3 leads to loss of the pituitary, whereas nuclei surrounding the SCN remain intact in *Six3<sup>flox/flox</sup>/Nestin-cre<sup>+</sup>* experimental animals. Bottom right inset in Otp column represents adjacent section for Six3 expression. **C**, Variability in the targeted elimination of floxed Six3 in E15.5 mice. Expression of Six3, Lhx1, and ROR $\alpha$  in *Six3<sup>flox/flox</sup>/Nestin-cre<sup>+</sup>* mice that showed either partial loss (top) or no loss (bottom) of Six3. Top, Unilateral Six3 deletions lead to unilateral loss of ROR $\alpha$  and Lhx1. Bottom, Mice that retain Six3 expression still express both Lhx1 and ROR $\alpha$ . Scale bars, 250  $\mu$ m.

**Supplemental Table 1.** Transcription factors with discrete expression within the mouse SCN at P0.

Gene	Entrez Gene ID	Family	SCN	Potentially in SCN	Boundary	In Situ Verified	Source	ABA E18 Expression Confirmation	ABA Adult Expression Confirmation
Sox2	20674	HMG	X					X	X
Sox5	20678	HMG	X						
Zfhx3	11906	Homeobox	X					X	X
Hmx3	15373	Homeobox	X						
Lhx1	16869	Homeobox	X			X		X	X
Six3	20473	Homeobox	X			X		X	
Six6*	20476	Homeobox	X			X	Jean et al., 1999		
Vax1	22326	Homeobox	X						X
Rora	19883	Nucrec	X			X		X	X
Rorb*	225998	Nucrec	X			X	Schaeren-Wiemers et al., 1997	X	X
Nr4a1	15370	Nucrec	X					X	X
Pou2f2*	18987	Pou	X			X	Rivkees et al., 1992	X	
Peg3	18616	ZN C2H2	X						
Tcf12	21406	bHLH		X					
Olig2	50913	bHLH		X					
Sox14	20669	HMG		X					
Dlx2	13392	Homeobox		X				X	X
Dlx5	13395	Homeobox		X					
Lhx9	16876	Homeobox		X					
Sox4	20677	HMG			X			X	
Dlx1	13390	Homeobox			X			X	
Isl1	16392	Homeobox			X			X	X
Lhx2*	16870	Homeobox				X	Menger et al., 2005	X	
Lhx5	16873	Homeobox			X			X	
Lhx8	16875	Homeobox			X			X	
Otp	18420	Homeobox			X	X		X	
Pbx3	18516	Homeobox			X			X	
Nr2f2	11819	Nucrec			X			X	

List of 28 transcription factors identified as within, potentially within, or directly adjacent to the postnatal mouse SCN obtained from re-examination of a genome-scale transcription factor expression database (Gray et al., 2004) and other published work (\*Rivkees et al., 1992; Schaeren-Wiemers et al., 1997; Jean et al., 1999; Menger et al., 2005). Genes highlighted in grey were further assessed for precise expression within the SCN region. The last two columns represent those genes whose expression was further confirmed in the Allen Brain Atlas either E18 (ABA E18) or Adult (ABA Adult) (Lein et al., 2007). bHLH – basic helix-loop-helix, HMG – high mobility group, Nucrec – nuclear receptor, ZN-Zinc finger type C2H2.

**Supplemental Table 2.** Transcription factors with discrete mRNA expression within the mouse anterior hypothalamus at E13.5.

Gene	Entrez Gene ID	Family	Anterior Hypothalamus	Boundary	In Situ Verified	Source	Database Expression Confirmation
Npas1	18142	bHLH	X				
Olig2	50913	bHLH	X				X
Baz2a	116848	Bromo	X				X
Baz2b	227940	Bromo	X				
Smarcc1	20588	Bromo	X				
Smarcd3	66993	Bromo	X				
FoxD3	15221	Forkhead	X				
FoxO1	56458	Forkhead	X				
Foxp4	74123	Forkhead	X				X
Hmgb1	15289	HMG	X				X
Hmgb2	97165	HMG	X				X
Sox21	223227	HMG	X				X
Sox4	20677	HMG	X				
Sox5	20678	HMG	X				X
Tef7l2	21416	HMG	X				X
Atbf1	11906	Homeobox	X				X
Dlx1	13390	Homeobox	X				X
Dlx2	13392	Homeobox	X		X		X
Dlx5	13395	Homeobox	X				X
Hes1	15205	Homeobox	X				X
Hmx2*	15372	Homeobox	X			Wang and Lufkin, 2000	X
Hmx3	15373	Homeobox	X				
HoxD3	15434	Homeobox	X				
Lhx1	16869	Homeobox	X		X		X
Lhx2	16870	Homeobox	X				X
Lhx4	16872	Homeobox	X				
Nkx2.1	21869	Homeobox	X				X
Six3	20473	Homeobox	X		X		X
Six6*	20476	Homeobox	X		X	Jean et al., 1999	X

**Supplemental Table 2 (Con't).** Transcription factors with discrete mRNA expression within the mouse anterior hypothalamus at E13.5.

Gene	Entrez Gene ID	Family	Anterior Hypothalamus	Boundary	In Situ Verified	Source	Database Expression Confirmation
Tcf2	21410	Homeobox	X				
Vax1	22326	Homeobox	X				X
Crip2	68337	LIM	X				X
Csrp2	13008	LIM	X				
Lmo4	16911	LIM	X				X
Zyx	22793	LIM	X				
Nr1h2	22260	Nucrec	X				X
Rxra	20181	Nucrec	X				
Nr2e1	21907	Nucrec	X				X
Pou2f2	18987	Pou	X		X		X
Rfx3	19726	RFX	X				X
Peg3	18616	ZN C2H2	X				X
Zic3	22773	ZN C2H2	X				X
Zic4	22774	ZN C2H2	X				
Chd4	107932	ZN PHD	X				
Sim1	20464	bHLH		X			
Baz1b	22385	Bromo		X			X
Cited1	12705	Cited		X			X
Dlx6	13396	Homeobox		X			X
Isl1	16392	Homeobox		X			X
Lhx5	16873	Homeobox		X			X
Otp	18420	Homeobox		X	X		X
Lmo1	109594	LIM		X			X
Rxrg	20183	Nucrec		X			
Nr2f2	11819	Nucrec		X			X
Pou3f1	18991	Pou		X			X
Pou3f2	18992	Pou		X			
Stat5A	20850	Stat		X			
Zdhhc2	70546	ZN DHHC		X			X

List of 58 transcription factors identified as within or directly adjacent to the SCN obtained from re-examination of a genome-scale transcription factor expression database (Gray et al., 2004) and other published work (\*Jean et al., 1999; Wang and Lufkin, 2000). Genes highlighted in grey were further assessed for precise expression within the SCN region. The last column represents those genes whose expression was further confirmed using either Genepaint (Visel et al., 2004) or Eurexpress (Diez-Roux et al., 2011). bHLH – basic helix-loop-helix, Bromo - Bromodomain, CITED – CREB-binding protein/p300-interacting transactivator with ED-rich tail HMG – high mobility group, Forkhead – Forkhead box, Nucrec – nuclear receptor, RFX – Regulatory factor X, STAT – signal transducer and activator of transcription, ZN C2H2 – Zinc finger type C2H2, ZN DHHC – Zinc finger DHHC domain-containing.

## **-CHAPTER 3-**

### **Role of retinal innervation and vasoactive intestinal polypeptide signaling in mouse suprachiasmatic nuclei development**

**Abstract**

The suprachiasmatic nuclei (SCN) can be both anatomically and functionally subdivided. It is unknown whether these subdivisions represent intrinsic genetic differences within cells or if they are induced through SCN maturation. Herein, we utilize mouse models that lack either *Atoh7*, necessary for retinal ganglion cell development, functional vasoactive intestinal polypeptide (VIP), or VIP receptor 2 (VPAC<sub>2</sub>, *Vipr2*) to test for the affects of two salient aspects of the circadian system in shaping SCN development. We found the size and location of the SCN to be unaffected by the absence of retinal innervation. In addition, animals lacking retinal innervation maintain localization in expression of *Six3*, *Lhx1*, and *Rora*, three transcription factors that mark the SCN. We found loss of VIP/VPAC<sub>2</sub> signaling had minimal effects on SCN size. Further, localization and expression levels of both *Six3* and *Lhx1* are maintained within animals kept under 12 h light/dark, while the expression of *Rora* became more consistent between SCN subdivisions. VIP and VPAC<sub>2</sub> mutant animals kept in constant darkness displayed greatly reduced levels of *Rora* while maintaining levels of *Six3* expression as compared to C57BL/6 controls. Despite reductions in the total levels of *Rora*, differences in expression between dorsal and ventral regions of the SCN remained, albeit to a lesser extent. The absolute difference between levels of *Rora* in SCN subdivisions does not correlate with behavioral rhythmicity in animals lacking VIP/VPAC<sub>2</sub> signaling. We conclude that maturational changes in the localization of transcription factors within the SCN occur independent of retinal innervation and VIP signaling and may reflect more intrinsic differences in subsets of neurons within the nuclei.



**Introduction**

Activity in the form of sensory stimuli, spontaneous activity, and neurotransmitter signaling has been shown to shape brain development (for review (Gu, 2002; Grubb and Thompson, 2004; Huberman et al., 2008; Butz et al., 2009). In the circadian system, ~20,000 neurons comprise the suprachiasmatic nuclei (SCN) that are responsible for producing and coordinating near 24 h rhythms for the entire body and entraining these rhythms to external stimuli. Two of the most salient aspects of the circadian system are retinal input, for daily and seasonal entrainment, and vasoactive intestinal polypeptide (VIP) signaling, for synchronization of individually oscillating cells (Aton et al., 2005). How these activity signals contribute to shaping the anatomical and functional development of the SCN is unknown.

The SCN undergoes modifications and refinements of its gene expression through development. Peptides, whose expression is known to distinguish two classic subdivisions of the SCN, core and shell, are localized to their corresponding regions from their onset (VanDunk et al., 2011). Unlike peptide expression, the adult localization of transcription factor (TF) expression occurs gradually and loosely correlates with SCN maturational time points. Specifically, *Lhx1* (Lim homeobox protein 1) expression becomes centralized within the SCN during the same perinatal period as peptide mRNA expression begins, approximately embryonic day 17.5 (E17.5) (VanDunk et al., 2011). In addition, expression of *Rora* (Retinoic orphan receptor alpha) becomes regionalized to the SCN shell concurrent with the increase in retinal innervation within the SCN as well

as an increase in the number of cells expressing VIP (approximately postnatal day seven to twenty-one (P7-P21)). The temporal expression patterns of immediate early genes *c-fos* and *pERK*, and well studied clock genes *Per1* and *Per2* in several species have pointed to at least two functionally distinct regions within the SCN: one of endogenous rhythmicity (shell) roughly correlating with areas of vasopressin expression and one of induced rhythms (core) roughly correlating with areas expressing VIP, gastrin-releasing peptide, or calbindin (mouse, rat: (Sumova et al., 1998; Guido et al., 1999; Hamada et al., 2001; Yan and Okamura, 2002; Ramanathan et al., 2006); hamsters: (Silver et al., 1996; Hamada et al., 2004)). The gradual localization of gene expression along with the presence of functionally distinct SCN subdivisions leads to questions of whether these changes and differential subdivision functions are intrinsic to the cells themselves or if they are induced.

Through sensory experience and early spontaneous waves of neural firing, retinal innervation and activity have been shown to shape adult patterns of connectivity and patterning in the formation of ocular dominance columns and lateral geniculate nucleus layer segregation, respectively (for review (Sengpiel and Kind, 2002; Del Rio and Feller, 2006)). Furthermore, neurotransmitters such as acetylcholine and serotonin aid in neurodevelopment by promoting cortical plasticity (for review (Gu, 2002)). Both modulation of sensory experience, loss of retinal inputs, and loss of VIP have been shown to impact circadian function. Light information entering the retina is processed by retinal ganglion cells which then transmit the photic and non-photoc information to several brain

regions, including the SCN by way of the retinohypothalamic tract. Both unilateral and bilateral enucleation in adult rats can lead to changes in the number of cells in the SCN expressing VIP (Holtzman et al., 1989; Okamoto et al., 1990; Laemle and Rusa, 1992; Denis et al., 1993). Moreover, enucleation in both rats and hamsters has revealed retinal innervation necessary for oscillations in pERK normally found within the core of the SCN (Lee et al., 2003). In addition, rearing animals under various photoperiods has a direct consequence on the establishment of free-running period both at the single cell and behavioral level (Ohta et al., 2006; Ciarleglio et al., 2011). In the circadian system loss of a principal neurotransmitter, VIP, leads to desynchronization of the individually oscillating cells (Aton et al., 2005). Thus, animals deficient in either VIP or VPAC<sub>2</sub> display altered behavioral rhythms, with most animals unable to maintain a normal single statistically significant circadian period (Harmar et al., 2002; Colwell et al., 2003). Currently, it is unknown whether retinal innervation or peptide signaling have a role in shaping SCN gene expression and/or cellular functioning.

Herein, we utilize complimentary genetic models to investigate the role of two critical circadian components in shaping the development of the SCN. Specifically, we monitor the localization of TF expression in mouse models that lack either *Atoh7*, necessary for retinal ganglion cell development, or functional VIP peptide or VIP receptor 2 (VPAC<sub>2</sub>, *Vipr2*). Understanding if retinal activity or signaling pathways contribute to changes in expression of SCN-specific TFs will help us gain insight into how functional subdivisions and ultimately the functional SCN network is formed.

**Materials and Methods**

*Animals and housing.* Male and female wild-type C57BL/6 (Charles River), Vip<sup>-/-</sup> (Colwell et al., 2003) (founders provided by Dr. Chris Colwell, UCLA), Vipr2<sup>-/-</sup> (Harmar et al., 2002) (founders provided by Chris Colwell, UCLA), and Atoh7Cre (Yang et al., 2003) (Susan Culican, Washington University in St. Louis) mice were used. Mice were maintained in a normal 12 h light/dark schedule under standard care conditions. Mutant animals were maintained on a C57BL/6 background (Charles River). All experiments were approved by the Animal Studies Committee at Washington University and followed NIH guidelines.

*Genotyping:* Mice were genotyped by PCR for presence of Atoh7, Vip, Vipr2 and/or wild-type alleles as previously described (Wee et al., 2002; Colwell et al., 2003), or as outlined by The Jackson Laboratory. The presence of Cre was determined by the presence or absence of an amplified band as previously described (VanDunk et al., 2011).

*Tissue acquisition.* Unless otherwise stated, all collections were done at approximately zeitgeber time (ZT) 8. Neonatal pups (P0-P2) from timed pregnant females (morning of plug = E0.5) were anesthetized on ice and transcardially perfused with 4% paraformaldehyde (PFA) in 0.1 M PBS, pH 7.4. Older mice were anesthetized with a ketamine/xylazine mixture before perfusion with 0.9% saline followed by 4% PFA. All tissues were post fixed in 4% PFA overnight at 4°C, cryoprotected in 20% sucrose in

PBS, frozen in O.C.T. Compound Embedding Medium (Tissue-Tek), and stored at -75°C. Serial sections (20µm) were cut on a Hacker cryostat and thaw mounted on Superfrost Plus slides (Thermo Fisher Scientific). Five adjacent sets of sections were prepared from each postnatal age and stored at -20°C.

*Probe synthesis.* Plasmids for *in situ* hybridization probes were acquired from published sources (Gray et al., 2004; Burns et al., 2008; Zhang et al., 2008) or purchased from Open Biosystems. Gene fragments from verified plasmids were linearized by direct amplification by sequence or vector-specific PCR. Digoxigenin (DIG)-labeled anti-sense and sense RNA probes were made using PCR products as template and T7, T3, or SP6 RNA polymerases (Roche). cRNA probes were purified using Quick Spin columns (Roche) and quantified by spectrophotometry. Probes were used at a concentration of 1-2 µg/ml. Sense counterparts of all probes were tested to ensure probe specificity.

*In situ hybridization.* Slides were immersed in 4% PFA, permeabilized with proteinase K, returned to 4% PFA before being washed in 0.1 M triethanolamine-HCl with 0.25% acetic anhydride. Once blocked in hybridization buffer at 65°C slides were incubated in hybridization buffer containing 1-2 µg/ml DIG-labeled anti-sense cRNA overnight at 65°C. Slides were then washed in 2XSSC buffer at 62°C, washed in 0.2XSSC at 65°C, blocked with 10% normal horse serum (NHS) in 0.1M PBS, and incubated in alkaline phosphatase labeled anti-DIG antibody (1:2000 in 10% NHS; Roche) overnight. Sections

were washed and color was visualized using Nitro blue tetrazolium and 5-Bromo-4-chloro-3-indolyl phosphate (Roche). Staining was stopped after visual inspection. Sections were washed, fixed in 4% PFA, and coverslipped in 90% glycerol, Vectashield Mounting Medium (Vector Laboratories), or UltraCruz Mounting Media with DAPI (Santa Cruz Biotechnology).

*Image acquisition.* Images were acquired using a Nikon Eclipse 90i microscope, Photometrics Coolsnap HQ2 camera with a Prior Scientific ProScan II motorized translation stage, and acquired in Volocity (PerkinElmer Life and Analytical Sciences). Images were exported as 8bit JPEG or TIFF files. All images were adjusted for clarity by filtering and/or modifying levels, as necessary, in Photoshop (Adobe Systems). To improve aesthetics of images, non-tissue backgrounds were processed to exclude debris.

*mRNA quantification and analysis.* The most mid-SCN coronal section for each brain was selected for gene expression quantifications by assessing rostral to caudal location as well as section with the greatest area. Percentage of SCN area containing gene expression was calculated by dividing area expressing mRNA by the total estimated SCN area marked with DAPI, where area was calculated by outlining regions of interest within NIH ImageJ. Whole SCN quantifications of mRNA expression were performed by quantifying intensity of staining relative to background from 10x ISH images using NIH ImageJ and are reported in arbitrary intensity units (AIUs). Margins of the SCN were determined by

cell density marked with DAPI nuclear stain. Subdivision specific measurements were made by first outlining the total SCN area as marked by DAPI stain. Areas within the dorsal or ventral SCN were sampled as representative of either shell or core subdivisions, respectively. The sample for ventral SCN was obtained from an area representing the bottom 1/3 and middle half of each individual nucleus. Within NIH ImageJ a ROI ellipse was drawn within the ventrally isolated region and measured, the same ROI circle was then placed within the dorsal SCN and a representative shell measurement was taken. Absolute intensity differences between SCN subdivisions were calculated by taking dorsal values minus ventral values. For all statistics and values listed in tables, left and right SCN nuclei from each animal were tested as individual samples, thus one brain produced two samples. The differences were analyzed using Student's unpaired  $t$  test.

General SCN morphology was assessed by obtaining outlines drawn based on cellular density marked with nuclear DAPI stain and measuring individual nuclei for mid-SCN width, area, and cell density. Rostral to caudal length of the nuclei was calculated by counting the number of sections containing Rora staining and estimating length based on section thickness.

*Behavioral recording.* Adult (P30+)  $Vipr2^{-/-}$  (n=9),  $Vip^{-/-}$  (n=9), and C57BL/6 mice (n=4) were housed individually in cages equipped with running wheels and maintained in light-tight chambers illuminated with fluorescent bulbs ( $2.4 \pm 0.5 \times 10^{18}$  photons  $s^{-1} m^{-2}$ ; General Electric). Running wheel activity was recorded in 6 min bins (Clocklab,

Actimetrics) during 6 days in 12 h light/dark and then during 9 days in constant darkness. Animals were collected at a projected CT6/8 based on individual locomotor periodicities. All animals were enucleated under red light before perfusion.

*Analysis of Behavioral Rhythmicity.* Behavioral rhythmicity was analyzed as described by Aton and colleagues (Aton et al., 2005). Designations for rhythmic behavioral categories were made in the following manner: rhythmic (single statistically significant period), polyrhythmic (two or more significant periods), or arrhythmic (no significant period).



## Results

### Effects of retinal innervation on SCN transcription factor expression

#### *Morphological Analysis*

Several methods have been used in the past to address the role of retinal activity in the development of the SCN. Enucleation and use of a naturally occurring anophthalmic mouse have produced varying effects on gross anatomical SCN development ranging from rostral displacement of nuclei (bilateral enucleation, (Nagai et al., 1992)), to severe loss in cell numbers and unilateral loss of nuclei (anophthalmic, (Silver, 1977); microphthalmic, (Tokunaga et al., 1997)). However, anophthalmic mice have a mutation in the early patterning gene, *Rx*, necessary for the formation of not only the eye but also components of forebrain (Mathers et al., 1997) which may produce additional affects leading to morphological changes. In order to understand the developmental role of retinal innervation in shaping the SCN we took advantage of a mouse deficient in a key TF expressed almost exclusively in the retina and necessary for ganglion cell development, *Atoh7* (also termed *Math5*) (Wang et al., 2001). *Atoh7* knock-out (*Atoh7*<sup>-/-</sup>) mice lack ~80% of retinal ganglion cells and fail to form both the optic and retinohypothalamic tracts (RHT). Lack of retinal input renders these animals unable to entrain to light and to have a slightly increased circadian period (Wee et al., 2002). *Atoh7* knock-outs and heterozygous littermates were kept on a 12 h light/dark cycle (LD) and sampled at mid-day on postnatal days two (P2; *Atoh7*<sup>+/-</sup> (n=2), *Atoh7*<sup>-/-</sup> (n=3)) and 21-25

(P21-P25; *Atoh7*<sup>+/-</sup> (n=4), *Atoh7*<sup>-/-</sup> (n=8)). In this manner both initial formation of the SCN, the subsequent increase in nuclei size, and general morphology could be assessed.

The SCN was easily recognized as bilateral densely packed clusters of cells on either side of the third ventricle in all animals assessed (Figure 1). Further, general rostrocaudal location (data not shown), coronal mid-SCN area, and rostral to caudal length of the SCN were similar between *Atoh7*<sup>+/-</sup> and *Atoh7*<sup>-/-</sup> animals at both P2 and P21-P25 (Table 1). In addition, density of cells within the P21-P25 mid-coronal SCN of *Atoh7*<sup>-/-</sup> animals was similar to *Atoh7*<sup>+/-</sup> littermates (Table 1). In summary, we find retinal innervation is not necessary for SCN localization, proliferation, or rostral to caudal nucleus expansion.

#### *In situ hybridization Analysis*

To examine the effects of retinal innervation on maturation of the SCN, *Atoh7*<sup>+/-</sup> and *Atoh7*<sup>-/-</sup> mice at both P2 and P21-P25 were assessed for whole SCN expression and localization of *Six3*, *Lhx1*, and *Rora*. Overall, we found maturation of *Lhx1* and *Rora* patterns of expression within the SCN occur independent of retinal innervation (Figure 2). The pan-SCN localization and level of *Six3* expression was maintained in P2 and P21-P25 *Atoh7*<sup>-/-</sup> mice as compared to *Atoh7*<sup>+/-</sup> and was not statistically different (P2: *Atoh7*<sup>+/-</sup>,  $29.6 \pm 0.95$  AIU, *Atoh7*<sup>-/-</sup>,  $36.6 \pm 4.25$  AIU;  $p=0.164$ ; P21-P25: *Atoh7*<sup>+/-</sup>,  $28.92 \pm 2.77$  AIU, *Atoh7*<sup>-/-</sup>,  $25.18 \pm 2.07$  AIU;  $p=0.299$ ) (P2, Figure 2A; P21-P25, Figure 2B).

Localization of Lhx1 to the central SCN was seen in Atoh7 knock-out animals at both P2 and P21-P25, but the percentage of SCN expressing Lhx1 was reduced in comparison to Atoh7 heterozygotes (P2: Atoh7<sup>+/+</sup>, 33.04 ± 1.9%, Atoh7<sup>-/-</sup>, 24.358 ± 4.03%;  $p=0.022$ ; P21-P25: Atoh7<sup>+/+</sup>, 34.84 ± 1.96%, Atoh7<sup>-/-</sup> 29.09 ± 2.88%;  $p=0.029$ ) (P2, Figure 2A; P21-P25, Figure 2B). Whole SCN levels of Lhx1 remained similar between Atoh7<sup>+/+</sup> and Atoh7<sup>-/-</sup> animals (P2: Atoh7<sup>+/+</sup>, 13.36 ± 3.06 AIU, Atoh7<sup>-/-</sup> 6.63 ± 3.93 AIU;  $p=0.09$ ; P21-P25: Atoh7<sup>+/+</sup>, 9.48 ± 1.38 AIU, Atoh7<sup>-/-</sup> 6.91 ± 2.25 AIU;  $p=0.18$ ).

Whole SCN levels of Rora expression in P2 Atoh7<sup>-/-</sup> mice were consistent with Atoh7<sup>+/+</sup> mice (Atoh7<sup>+/+</sup>, 17.6 ± 0.68 AIU, Atoh7<sup>-/-</sup> 21.75 ± 3.6 AIU;  $p=0.306$ ). However, at P21-P25 Atoh7 knock-outs displayed a reduction in whole SCN levels of Rora (Atoh7<sup>+/+</sup>, 33.07 ± 2.00 AIU, Atoh7<sup>-/-</sup> 19.52 ± 2.70 AIU;  $p=0.001$ ) (P2, Figure 2A; P21-P25, Figure 2B). To specifically address the localization of Rora to the SCN shell the absolute intensity difference between dorsal and ventral SCN were quantified and compared between Atoh7<sup>+/+</sup> and Atoh7<sup>-/-</sup> mice (see methods). We find that the absolute intensity differences between dorsal and ventral levels of Rora are comparable to heterozygous controls for two of eight Atoh7 knock-outs tested (Figure 2B). This suggests that in the absence of retinal innervation the localization of Rora and Lhx1 mRNA can be achieved.

### **Effects of VIP/VPAC<sub>2</sub> signaling on SCN transcription factor expression**

Disassociated SCN cultures from mice lacking VIP show that individual cells become desynchronized with one another and can re-synchronize with bath application of a Vipr2 agonist (Aton et al., 2005). Further, animals deficient in VIP or VPAC<sub>2</sub> and kept in constant darkness (DD) display a range of behavioral phenotypes. The behavioral inconsistencies are thought to be a consequence of lower coherence of individually oscillating cells leading to lower amplitude network oscillations (Ciarleglio et al., 2009). It is not known whether VIP/ VPAC<sub>2</sub> signaling plays a role in the development of the SCN in addition to acting as a synchronizing agent. Recent research suggests VIP and VPAC<sub>2</sub> may have a role in the regulation of gating of cellular response to light. The absence of VIP and VPAC<sub>2</sub> shifts the time of day in which cells of the SCN can respond to a light pulse, from being gated only during subjective night periods to responding to light pulses given both during subjective day and subjective night (Hughes et al., 2004; Dragich et al., 2010). Thus, it is possible that the onset of VIP expression or VIP signaling may elicit changes in single cells altering cellular properties and ultimately changing functionality or inducing expression of specific TFs.

### *Morphological Analysis*

We find that the SCN remain present as bilateral nuclei and in similar rostrocaudal locations in animals either lacking VIP or VPAC<sub>2</sub> when compared to C57BL/6 (Wt) controls. Vip<sup>-/-</sup> and Vipr2<sup>-/-</sup> animals displayed similar rostral to caudal

SCN length, mid-SCN width, and cell density; however a reduction in mid-SCN area was observed in both  $Vip^{-/-}$  and  $Vipr2^{-/-}$  animals when compared to Wt (Figure 3, Table 2).

#### *In situ hybridization Analysis*

The role of intrinsic peptide activity in SCN development was assessed by examination of  $Vip^{-/-}$  and  $Vipr2^{-/-}$  mice under LD (collected at ZT8) and DD (collected at projected CT6) conditions. Tissue was examined for the expression and localization of *Rora* and *Six3* under both light conditions, and *Lhx1* under LD.

#### *LD*

$Vip^{-/-}$  (n=5) animals maintained in LD show levels of *Six3* gene expression similar to that of  $Vip^{+/-}$  littermates (n=2) ( $12.658 \pm 1.039$  and  $13.53 \pm 1.481$  respectively,  $p=0.201$ ) (Figure 4A). In addition, there were no changes in the whole SCN level of *Lhx1* expression or percentage of SCN expressing *Lhx1* (Figure 4A). Whole SCN levels of *Rora* were also found comparable between  $Vip^{-/-}$  and  $Vip^{+/-}$  ( $Vip^{-/-}$ ,  $14.222 \pm 1.246$ ;  $Vip^{+/-}$ ,  $20.324 \pm 3.72$ ;  $p=0.638$ ) animals. The absolute intensity difference between *Rora* levels in the dorsal and ventral SCN were reduced in both  $Vip^{+/-}$  and  $Vip^{-/-}$  mice as compared to values obtained from Wt animals kept in LD (Previous observations). No difference was seen in the absolute intensity differences of *Rora* in SCN subdivisions between  $Vip^{+/-}$  and  $Vip^{-/-}$  animals (Figure 4A).

*DD*

Expression of *Six3* was unaffected in VIP/VPAC<sub>2</sub> signaling mutants kept in DD when compared to Wt, (Wt,  $19.93 \pm 1.18$  AIU; *Vip*<sup>-/-</sup>,  $19.36 \pm 1.03$  AIU,  $p=0.788$ ; *Vipr2*<sup>-/-</sup>,  $19.18 \pm 0.92$  AIU,  $p=0.875$ ). However, *Vip*<sup>-/-</sup> and *Vipr2*<sup>-/-</sup> animals displayed significantly reduced whole SCN levels of *Rora* (Wt,  $25.47 \pm 1.77$  AIU; *Vip*<sup>-/-</sup>,  $15.36 \pm 0.58$  AIU,  $p=0.005$ ; *Vipr2*<sup>-/-</sup>,  $15.47 \pm 0.92$  AIU,  $p=0.008$ ). There were no significant differences between levels of whole SCN *Rora* in *Vip*<sup>-/-</sup> compared to *Vipr2*<sup>-/-</sup> mice, suggesting that either method of disrupting signaling reduces the total expression of *Rora* at CT6/8.

Wild-type animals displayed large absolute intensity differences in expression of *Rora* between SCN subdivisions ( $10.73 \pm 2.2$  AID). Reduced whole SCN levels of *Rora* represented reductions in both ventral and dorsal levels in both VIP and VPAC<sub>2</sub> mutants. Despite these reductions, differences between levels of *Rora* in dorsal and ventral regions of the SCN remained with higher levels in the dorsal SCN maintained, albeit to a lesser extent (*Vip*<sup>-/-</sup>,  $2.4 \pm 0.5$  AID; *Vipr2*<sup>-/-</sup>,  $6.66 \pm 1.4$  AID).

It is currently unknown whether the regionalization of *Rora* to the shell of the SCN, leading to higher levels of *Rora* in the dorsal than in the ventral SCN, is necessary for behavioral rhythmicity. *Vip*<sup>-/-</sup> and *Vipr2*<sup>-/-</sup> animals kept in DD show variability in their ability to produce free-running locomotor rhythms. Behavioral variations include animals with a single, slightly shortened locomotor circadian period, presence of two or more circadian periods, and some mice which are completely arrhythmic (Harmar et al., 2002;

Colwell et al., 2003; Hughes and Piggins, 2008). It is currently unknown whether there are intrinsic differences between animals deficient in either VIP or VPAC<sub>2</sub> that are able to retain a single period and those that are incapable of producing a single stable rhythm.

To address whether localization or abundance of Rora expression and behavioral phenotype were correlated we analyzed behaviorally segregated *Vip*<sup>-/-</sup> and *Vipr2*<sup>-/-</sup> animals. Free-running behavior was observed over a period of nine days in DD after which each animal was assigned as rhythmic (single statistically significant period), polyrhythmic (two or more significant periods), or arrhythmic (no significant period). Behavioral segregation yielded *Vip*<sup>-/-</sup> and *Vipr2*<sup>-/-</sup> animals within each category (*Vip*<sup>-/-</sup>: 4 Rhythmic, 1 Polyrhythmic, 3 Arrhythmic; *Vipr2*<sup>-/-</sup>: 1 Rhythmic, 3 Polyrhythmic, 4 Arrhythmic) (*Vip*<sup>-/-</sup> Figure 5A, *Vipr2*<sup>-/-</sup> Figure 5B). We found that regardless of their behavioral rhythmicity, all VIP and VPAC<sub>2</sub> mutant groups differed significantly from Wt in whole SCN levels of Rora. There was no correlation between the animals with the highest whole SCN Rora levels and behavioral status. In addition, there was no correlation between the animals with the highest absolute intensity differences between SCN subdivisions and behavioral rhythmicity, although *Vipr2*<sup>-/-</sup> animals displayed consistently higher subdivision differences in Rora than *Vip*<sup>-/-</sup> animals (*Vip*<sup>-/-</sup> Figure 5A, *Vipr2*<sup>-/-</sup> Figure 5B). It remains unclear whether there is a connection between the retained ventral vs. dorsal distinctions in Rora expression and rhythmicity or if the variations are merely a consequence of random desynchrony.

**Discussion:**

Proper development of complex neural systems involves the coordination of both intrinsic genetic factors and extrinsic signals (Gu, 2002; Grubb and Thompson, 2004; Huberman et al., 2008; Butz et al., 2009). Within the circadian system two key elements, retinal input, and VIP signaling are crucial for proper system function. However, the roles for these elements in the appropriate development of the SCN are unknown. Herein, we show that maturational changes in localization of gene expression occur independent of retinal innervation or VIP signaling, suggesting that these changes may reflect more intrinsic differences within subsets of SCN neurons.

Atoh7 knock-outs, due to a loss of retinal ganglion cells, do not transmit light information to the SCN and animals lose their abilities to entrain to light cycles. We found that Atoh7 mutant mice show no changes in SCN structure or location. Further, we found no changes in the centralization of Lhx1 or the pan-SCN expression of Rora and Six3 at P2. The collection of P21-P25 free-running mice at a single ZT offers a pseudo-randomized sampling of animals throughout a circadian time frame. Sampling animals with free running molecular clocks means that while Rora is expressed in a circadian fashion (Sato et al., 2004) individual animals may be at different points within mRNA oscillations. If retinal innervation was necessary for variations in subdivision specific Rora expression we would expect there to never be changes in expression between the subdivisions, expressed either as pan-SCN Rora expression or low levels of Rora throughout the nuclei. We found that distinctions are still present between ventral and



dorsal levels of *Rora* in a subset of *Atoh7* mutant animals. In addition, we found centralization of *Lhx1* and the pan-SCN expression of *Six3* are retained at this later postnatal stage.

While *Lhx1* expression remained centralized in *Atoh7* knock-out animals at both P2 and P21-P25 the area of SCN expressing *Lhx1* was reduced. Interestingly, whole SCN levels of *Lhx1* remained, suggesting a smaller number of cells expressing higher levels of *Lhx1* in the absence of retinal innervation. It is currently unclear why maintaining total levels of *Lhx1* expression may be important for the SCN.

VIP signaling mutants showed no changes in the location or presence of SCN nuclei. We found that while rostral to caudal SCN length and mid-SCN width remained similar, there was a significant difference in mid-SCN area of *Vip*<sup>-/-</sup> and *Vipr2*<sup>-/-</sup> mice compared to wild-type controls. Further investigations will need to be carried out in order to see if this represents a true change in the absence of VIP signaling or is a consequence of uncontrollable variations in section angle from sample to sample.

It has been well established that animals lacking VIP/ VPAC<sub>2</sub> signaling maintain behavioral rhythmicity in the presence of 12 h light/dark cycles by processes of negative and positive masking (Harmar et al., 2002; Colwell et al., 2003). The mechanisms of how light directly influences locomotor activities are unknown. Regardless of their behavioral abilities in response to light, several groups have shown reduced clock gene expression in VIP/ VPAC<sub>2</sub> signaling mutants kept under both LD and DD conditions. Levels of clock gene expression in *Vipr2*<sup>-/-</sup> mice and SCN slice preparations show reductions in levels of

clock genes *Per1*, *Per2*, and *Bmal1* in *Vip*<sup>-/-</sup> (Loh et al., 2011) and *Per1*, *Per2* in *Vipr2*<sup>-/-</sup> mice (Harmar et al., 2002). Generally, the reductions in clock gene expression in animals kept without light cues are thought to reflect desynchrony among individual cellular oscillators throughout the SCN and possibly also the loss of a small number of rhythmically active cells (Aton et al., 2005). We find that VIP mutants kept in DD display severely reduced levels of *Rora*, but retain similar levels of expression as heterozygotes when under an LD cycle. This suggests that the masking effects may not be acting completely indirectly, but that there may be some connection between the mechanism of masking and the ability to coordinate rhythms within the SCN on a cellular level. However, we must take into account that VIP heterozygous animals have been shown to display an intermediate phenotype in disruption of period and phasing of neuronal activity rhythms (Brown et al., 2007). This may indicate that there is an intermediate effect on the number of cells synchronized or the level of synchronization that may lead to a reduction in total levels of *Rora* expression seen at one given time.

Similar to VIP mutants, we find VPAC<sub>2</sub> mutant animals also show reduced levels of *Rora* within whole, ventral, and dorsal SCN measurements in DD as compared to wild-type. Interestingly, the absolute differences in *Rora* expression between dorsal and ventral SCN in VPAC<sub>2</sub> mutants were statistically similar to wild-type animals, while VIP mutants show statistically significant differences when compared to both wild-type and VPAC<sub>2</sub> animals.

Surprisingly, no differences were found between the expression of *Six3* in VIP heterozygous and homozygous mutants kept in LD or homozygous VIP and VPAC<sub>2</sub> mutants kept in DD as compared to wild-type animals. The absence of reduction in whole SCN expression of *Six3* in VIP and VPAC<sub>2</sub> mutants kept in DD suggests that either expression of *Six3* is independent of the molecular oscillations of the clock or that regulation of *Six3* expression is mediated by light input and is not circadianly regulated.

Not all VIP and VPAC<sub>2</sub> mutants are behaviorally arrhythmic. A subset of these animals are able to maintain a single circadian period, although shortened (Colwell et al., 2003). It is not known whether this ability to maintain rhythmicity reflects unique properties of the network changed with loss of VIP, compensation from an additional signaling element, random coherence, or maintenance of aspects of functional subdivisions. In wild-type animals *Rora* is expressed at significantly higher levels in the shell than in the core. It is not known if this pattern has behavioral relevance. We found that rhythmic, arrhythmic, and polyrhythmic VIP and VPAC<sub>2</sub> mutants had reduced levels of *Rora* expression in the ventral and dorsal SCN, with higher levels of *Rora* in the dorsal SCN maintained in most animals. There was no correlation between either the total levels of *Rora* expression or the amount of absolute difference in *Rora* expression between subdivisions and behavioral locomotor outcome. Thus, it is likely that these changes are merely a consequence of non-uniform desynchrony occurring within SCN nuclei of individual animals.

Overall, we found that the localization of TFs within the SCN, namely centralization of *Lhx1* and the shell regionalization of *Rora*, occur independent of retinal innervation and VIP signaling. This may provide evidence of more intrinsic differences between specific subsets of SCN neurons. However, it is possible that changes in TF localization may still indicate functional time points induced by means other than those tested here such as progression of synaptogenesis, gliogenesis, etc. Future work will need to be done to continue finding and understanding the ways in which the SCN progresses and matures to form functional subdivisions and a functional nucleus.

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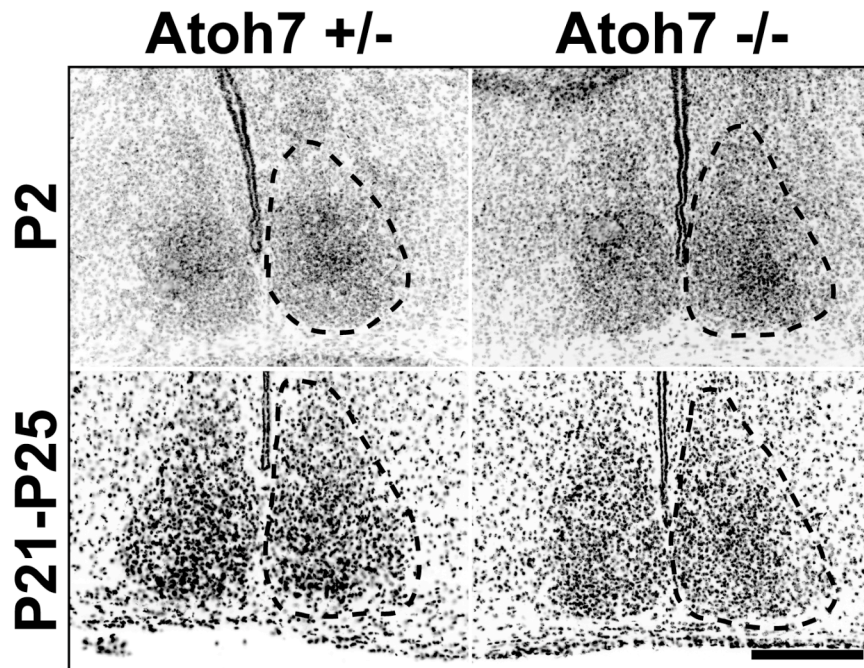
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**Figure 1.** SCN presence and location are unaffected by absence of retinal innervation. Inverted fluorescent DAPI stain of coronal mid-SCN at P2 and P21-P25 for *Atoh7*<sup>+/+</sup> and *Atoh7*<sup>-/-</sup> mice showing cellular density. Dotted lines indicate boundaries of the nuclei based off of DAPI stain. Scale bar, 250  $\mu$ m. Distinct bilateral nuclei representing the SCN are present along the ventral surface of the brain in *Atoh7* heterozygotes that possess an intact retinohypothalamic tract and *Atoh7* knock-outs lacking retinal innervation to the SCN.

**Table 1.** SCN area and length are maintained in the absence of retinal innervation.

Age, Genotype, n	Average mid-SCN Area (square micron)	Rostral to Caudal Length (um)	mid-SCN width (um)	Whole mid-SCN density (nuclei/ square micron)
<b>P2, Atoh7 +/-, (4)</b>	84,332 ± 4,283	320 ± 0.0	316.82 ± 14.99	-----
<b>P2, Atoh7 -/-, (6)</b>	76,617 ± 4,986 <i>p</i> =0.275	270 ± 50.00 <i>p</i> =0.500	331.77 ± 8.94 <i>p</i> =0.430	-----
<b>P21-P25, Atoh7 +/-, (8)</b>	87,663 ± 9,477	445 ± 47.87	284.59 ± 11.44	0.00886 ± 0.000530
<b>P21-P25, Atoh7 -/-, (16)</b>	99,271 ± 3,600 <i>p</i> =0.293	345 ± 16.37 <i>p</i> =0.125	326.54 ± 7.02 <i>p</i> =0.012	0.00792 ± 0.000278 <i>p</i> =0.160

Table displays mid-SCN area (calculated by averaging measurements of right and left nuclei for each animal), rostral to caudal length, mid-SCN width, and mid-SCN density measurements of SCN nuclei ( $\pm$  s.e.m.), showing no overall changes in SCN size in the absence of retinal innervation.

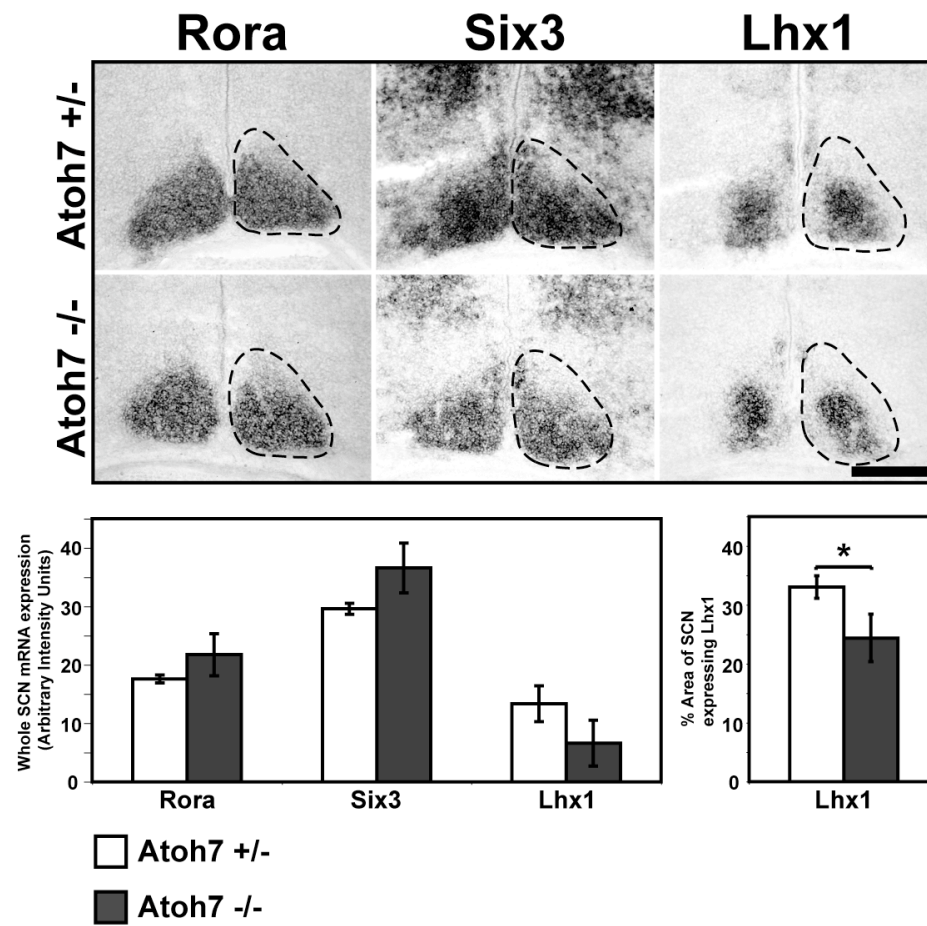


FIGURE 2A

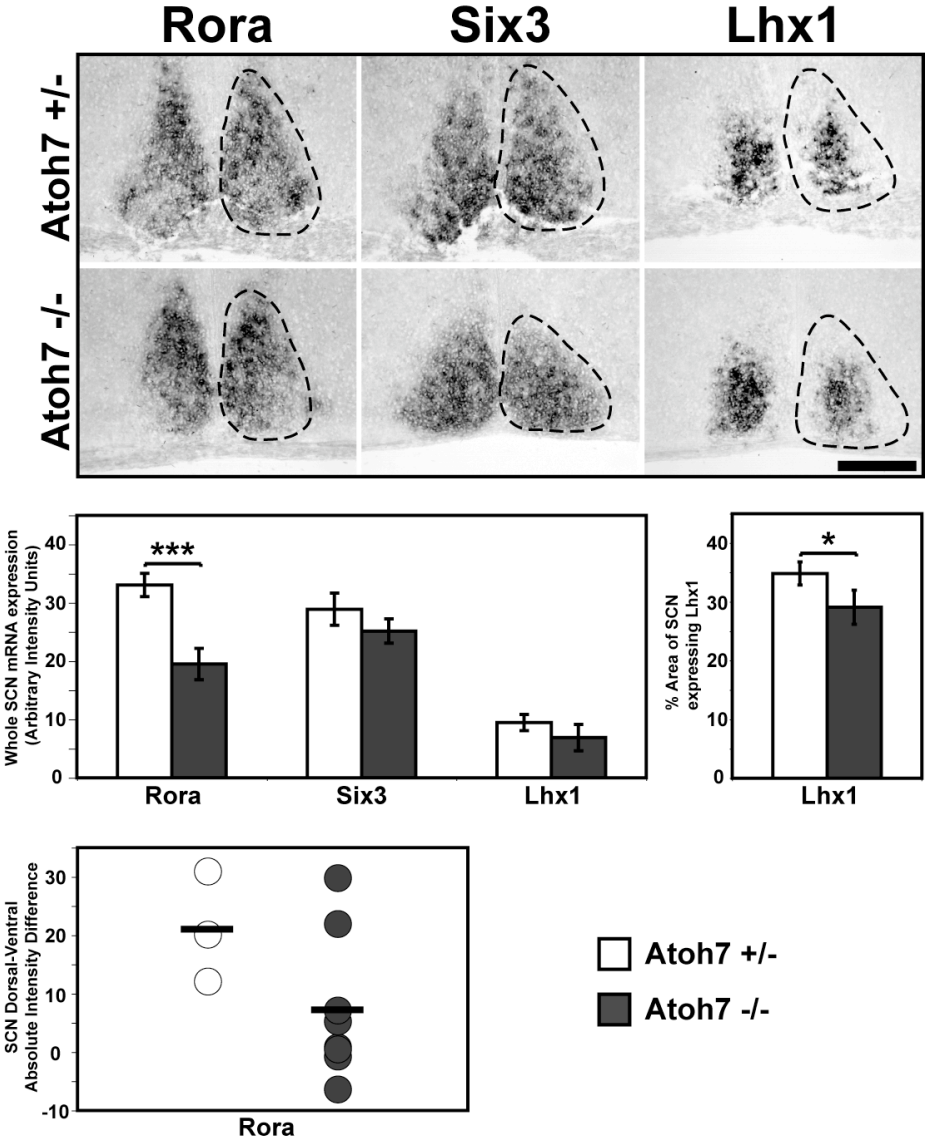
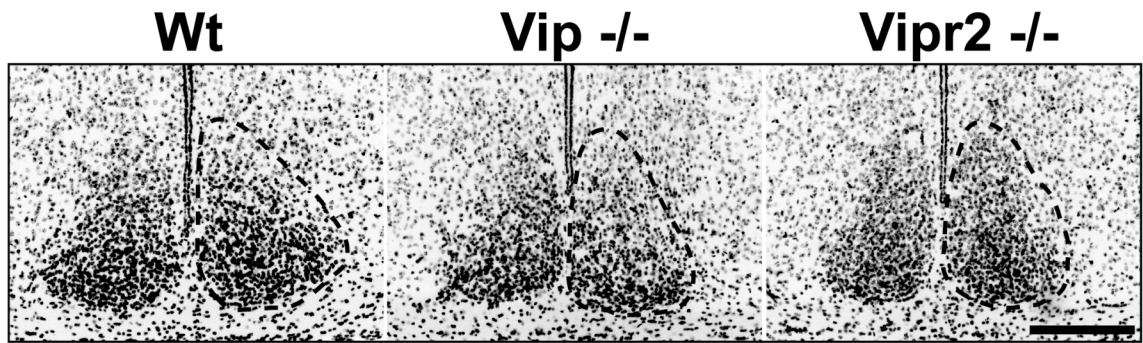


FIGURE 2B

**Figure 2.** Progressive localization of transcription factor expression is maintained in *Atoh7* knock-outs. *In situ* hybridization (ISH) of coronal mid-SCN expressing *Rora*, *Six3*, or *Lhx1* mRNA in P2 (**A**) and P21-P25 (**B**) *Atoh7*<sup>+/-</sup> and *Atoh7*<sup>-/-</sup> mice. Dashed lines indicate SCN outline based on DAPI nuclear stain. **A**, *top*, Representative images show pan-SCN expression of *Rora* and *Six3* as well as centralization of *Lhx1* maintained in *Atoh7* knock-out animals. *Bottom*, bar graphs show maintained whole SCN expression levels of *Rora*, *Six3*, and *Lhx1* measured in arbitrary intensity units (AIUs) (left) while percentage of SCN expressing *Lhx1* is reduced (right). **B**, *top*, ISH images depict maintained ability of *Atoh7* knock-outs to localize expression of *Rora* to the shell and *Lhx1* to the central SCN while conserving *Six3* expression throughout the nuclei. *Middle*, bar graph, indicates a significant reduction in *Atoh7* knock-out whole SCN levels of *Rora* as compared to *Atoh7* heterozygotes (left) and reduction in percentage of SCN expressing *Lhx1* (right). *Bottom*, scatter plot shows the absolute intensity difference in expression levels of *Rora* between SCN subdivisions and indicates no change between *Atoh7* knock-outs and heterozygotes. Wide black bars indicate average values. Scale bar, 250  $\mu$ m. Asterisks indicate significance based on un-paired *t* test (\*,  $p \leq 0.05$ ; \*\*,  $p \leq 0.01$ , \*\*\*,  $p \leq 0.005$ ).





**Figure 3.** SCN presence and location are unaffected by absence of VIP/ VPAC<sub>2</sub> signaling. Inverted fluorescent DAPI nuclear stain of coronal mid-SCN for C57BL/6 (Wt), *Vip*<sup>-/-</sup>, and *Vipr2*<sup>-/-</sup> show clear bilateral nuclei. Dotted lines indicate boundaries of the nucleus based off of DAPI stain. Scale bar, 250  $\mu$ m.

**Table 2.** SCN location and morphology are maintained in VIP/ VPAC<sub>2</sub> signaling mutants.

Age, Genotype, n	Average mid-SCN Area (square microns)	Rostral to Caudal Length (um)	mid-SCN width (um)	Whole mid-SCN density (nuclei/ square micron)
P21+, Wt, (8)	106,693 ± 4,264	470 ± 28.87	303.07 ± 9.09	0.00774 ± 0.00364
P21+, Vip <sup>-/-</sup> , (18)	91,413 ± 2,061 <i>p</i> =0.008	387 ± 16.67 <i>p</i> =0.053	301.80 ± 3.74 <i>p</i> =0.914	0.00779 ± 0.000184 <i>p</i> =0.898
P21+, Vipr2 <sup>-/-</sup> , (18)	90,873 ± 3,975 <i>p</i> =0.013	445 ± 25.00 <i>p</i> =0.532	294.68 ± 10.53 <i>p</i> =0.553	0.00816 ± 0.000235 <i>p</i> =0.353

Table displays mid-SCN area (calculated by averaging measurements of right and left nuclei for each animal), rostral to caudal length and mid-SCN width measurements of SCN nuclei ( $\pm$  s.e.m.). No changes were seen in rostral to caudal SCN length, SCN width, or mid-SCN cell density; however a slight difference was found in the mid-SCN area of Vip<sup>-/-</sup> and Vipr2<sup>-/-</sup> animals as compared to controls.

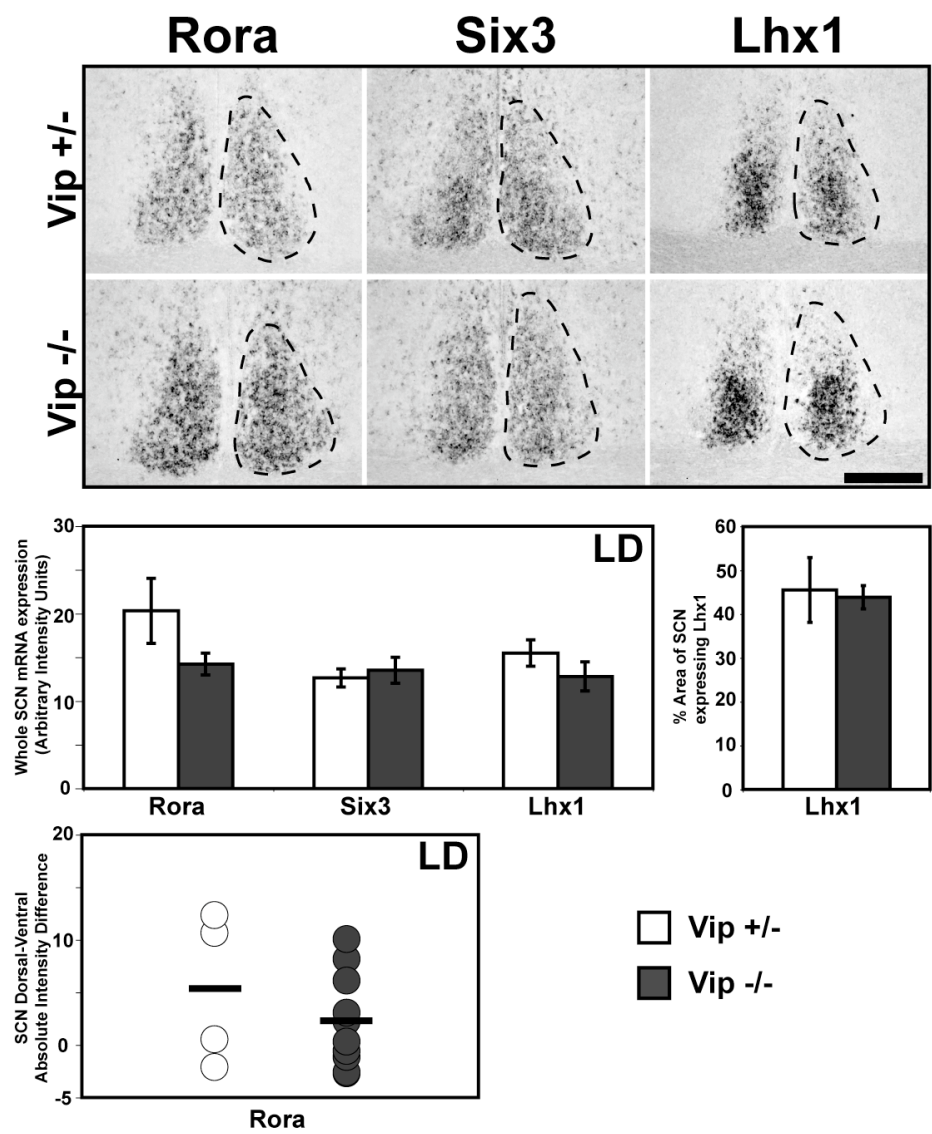


FIGURE 4A

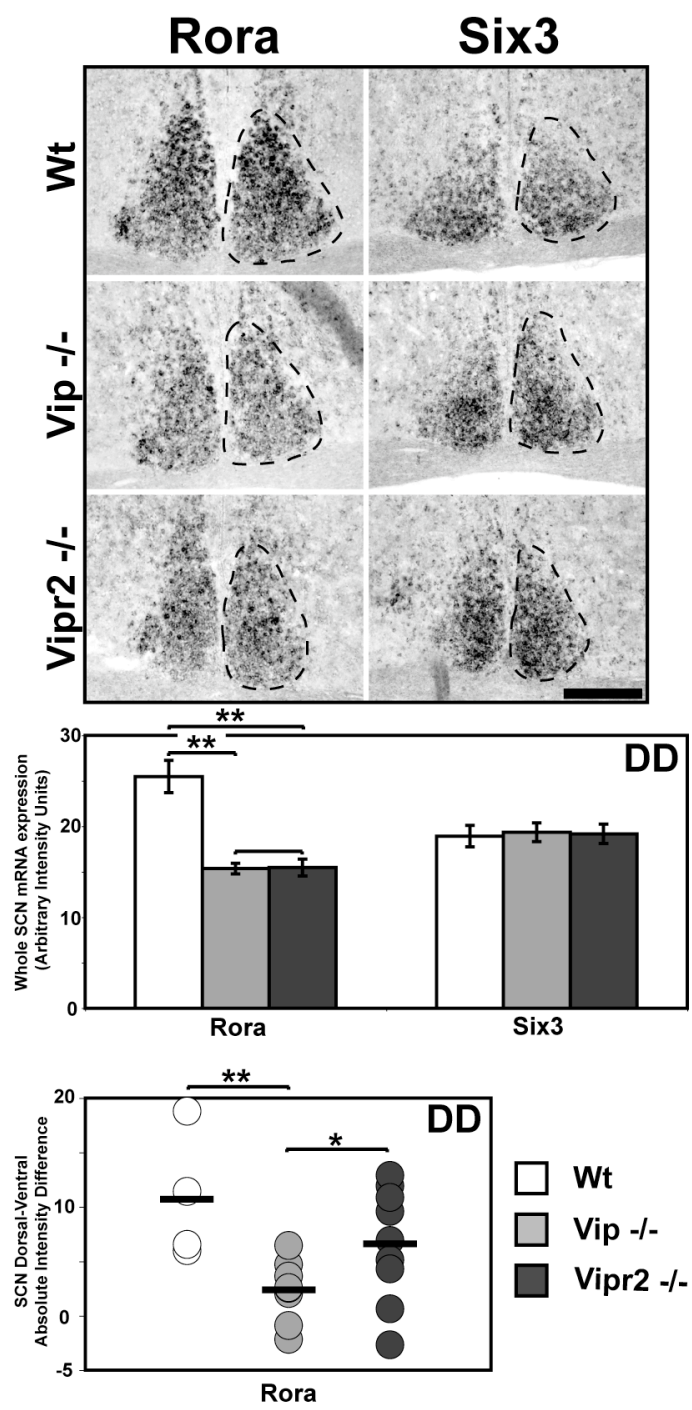


FIGURE 4B

**Figure 4.** Abundance of Rora expression is reduced in VIP and VPAC<sub>2</sub> mutants kept in constant darkness but not under 12 h light/dark. *In situ* hybridization (ISH) of coronal mid-SCN tissue from VIP and/or VPAC<sub>2</sub> knock-out animals and C57BL/6 (Wt) controls kept under 12 h light/dark (LD) (**A**) or constant darkness (DD) (**B**). **A**, *top*, ISH images depict maintained centralization of Lhx1 in Vip<sup>-/-</sup> animals. *Middle*, bar graphs indicate no differences between whole SCN expression of Rora, Six3, Lhx1 (left) and no change in the percentage of SCN expressing Lhx1 (right). *Bottom*, scatter plot shows no change in absolute intensity differences in Rora between SCN subdivisions. Wide black lines in scatter plots indicate average values. **B**, *top*, ISH images show lack of differential Rora expression between SCN subdivisions in VIP/ VPAC<sub>2</sub> signaling mutants. *Middle*, bar graph shows reduced expression of Rora in Vip<sup>-/-</sup> and Vipr2<sup>-/-</sup> animals. *Bottom*, Scatter plot depicts reductions in Rora absolute intensity differences between SCN subdivisions. Scale bars, 250  $\mu$ m. Asterisks indicate significance based on un-paired *t* test (\*,  $p \leq 0.05$ ; \*\*,  $p \leq 0.01$ , \*\*\*,  $p \leq 0.005$ ).

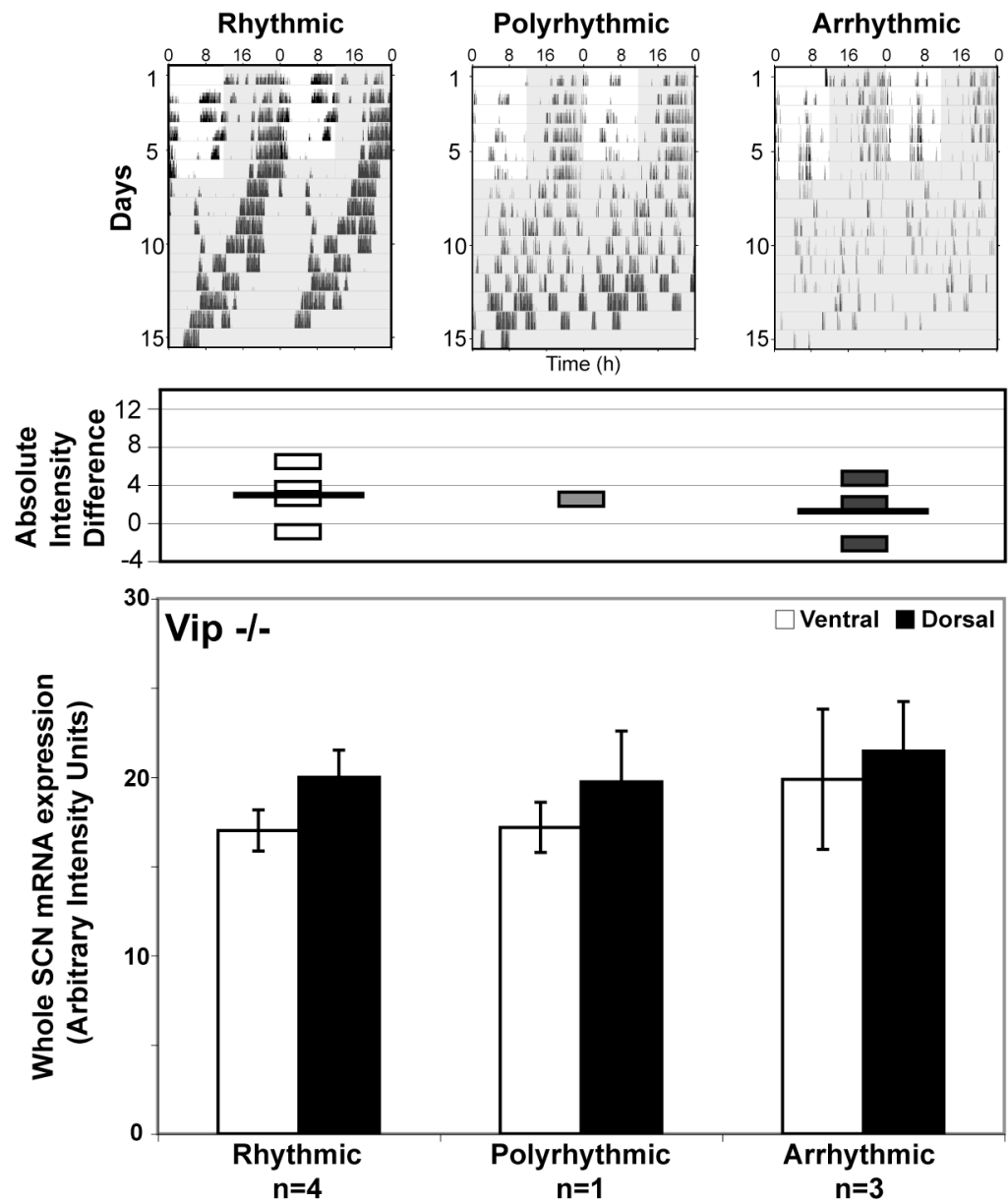


FIGURE 5A

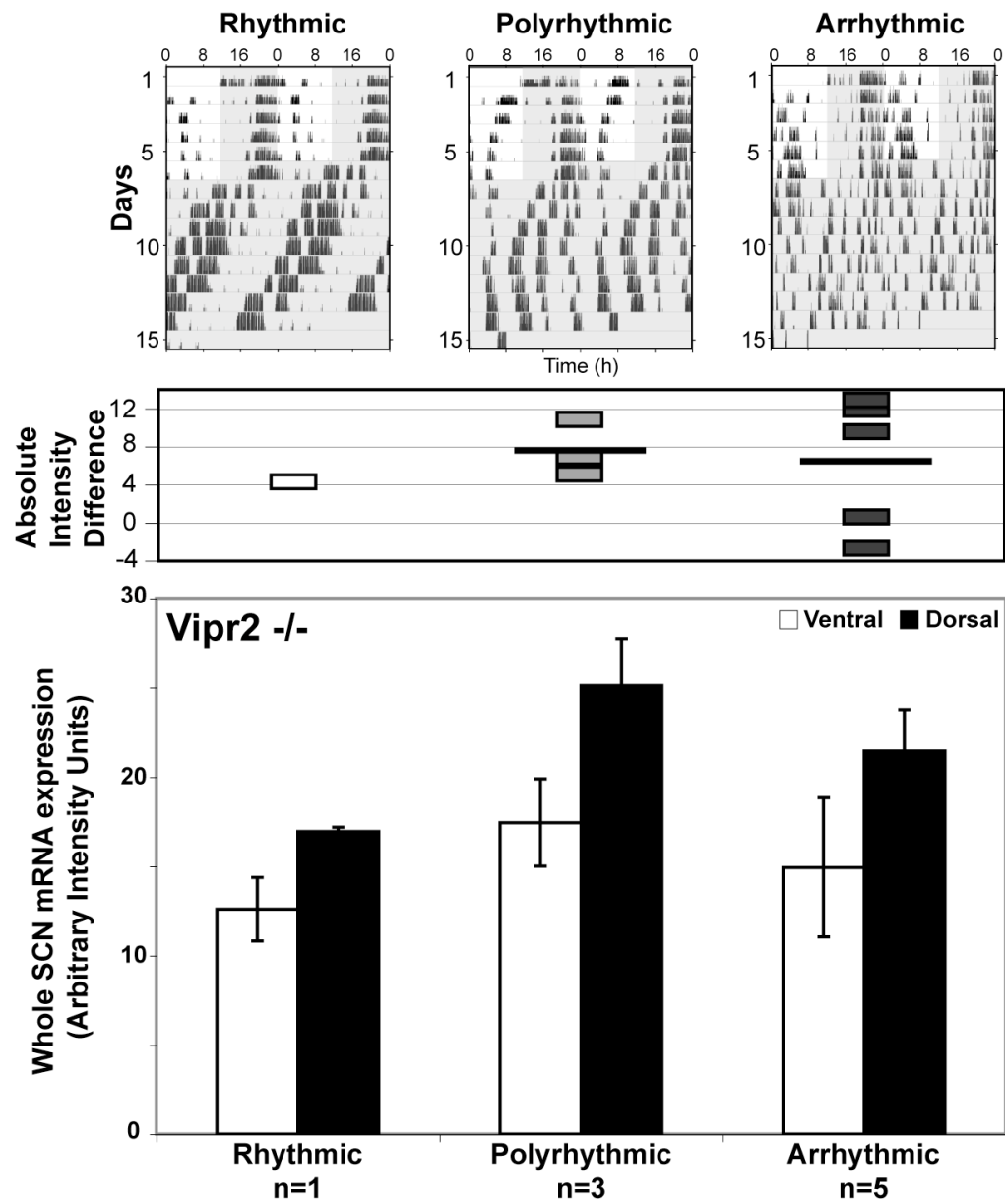


FIGURE 5B

**Figure 5.** Abundance and localization of Rora expression does not correlate with behavioral rhythmicity. **Top**, double-plotted actograms show wheel-running activity from either *Vip*<sup>-/-</sup> (**A**) or *Vipr2*<sup>-/-</sup> (**B**) animals maintained on a 12 h light/dark schedule for six days before being transferred to nine days in constant darkness. Grayed hours indicate periods of darkness. Animals were classified as behaviorally rhythmic, polyrhythmic, or arrhythmic based on their free-running rhythms. **Middle**, scatter plots display absolute intensity differences in the expression of Rora between SCN subdivisions. Wide black lines in scatter plots indicate average values. **Bottom**, bar graphs show average mRNA intensity of Rora in the ventral and dorsal SCN of behaviorally segregated mutants. Note that higher levels of Rora in the shell were maintained in all groups tested, but there is no correlation between levels of Rora and locomotor phenotype.



**-CHAPTER 4-**

**Roles of homeobox protein Six3 in postmitotic GABA-ergic  
populations of *Mus musculus***

## Abstract

The suprachiasmatic nuclei (SCN) act as principal mammalian circadian pacemakers regulating many biological processes including hormone secretion, body temperature regulation, and sleep/wake activity. Remarkably, the genes and transcription factors (TFs) necessary for the specification and maturation of the SCN have not been well defined. The TF, Six3, has recently been identified as expressed within SCN neurons from before initial specification to adult. The extended expression of Six3 in the SCN is not unique; rather Six3 remains expressed in several hypothalamic and thalamic nuclei even in adult mice. The postmitotic role for Six3 in neurons is unknown. Herein, we examine the effects of deletion of Six3 postmitotically in mice with emphasis on the SCN, by targeting deletion from postmitotic GABA-ergic populations. Within the SCN, successful Six3 deletion did not effect the expression and localization of TFs *Rora*, *Lhx1*, and *Per1* or the peptides vasoactive intestinal polypeptide or arginine vasopressin. *Six3<sup>flox/flox</sup>/Gad2-cre(+)* knock-outs displayed a severe growth deficit evident by postnatal day ten and leading to early lethality shortly after weaning. Six3 expression is unchanged in the pituitary gland but is completely eliminated from several GABA-ergic hormone-regulating nuclei including the arcuate and the substantia nigra pars reticulata. We conclude that the postmitotic deletion of Six3 from SCN neurons does not affect the phenotypic specification and localization of several SCN TFs or peptides, but may control the specification or function of nuclei responsible for key growth signals.

**Introduction**

The embryonic telencephalon/diencephalon gives rise to key structures regulating biological homeostasis and arousal as well as structures relaying and processing sensory information (McCormick and Bal, 1994; Williams et al., 2001). Currently, the molecular pathways controlling the development and function of these vital brain regions are not well understood. In order to elucidate the roles specific genes play in the development of behaviorally important structures, several groups have performed large-scale transcription factor screens, identifying genes expressed in relatively discrete populations of cells throughout the brain (Gray et al., 2004; Lein et al., 2007; Shimogori et al., 2010).

The SCN, central circadian pacemakers for the mammalian system, are just one of the many nuclei derived from the telencephalon/diencephalon. They are responsible for creating and maintaining near 24 h rhythms, which regulate essential homeostatic processes such as temperature and energy regulation (for review (Ko and Takahashi, 2006)). The SCN are heterogeneous nuclei comprised of equally rhythmogenic neurons, which vary in neuropeptide content, TF expression, response to stimuli, and other respects. Currently, the TFs that contribute to the specification of rhythmic neurons or cellular subtypes are unknown. Screens for TF expression have been successful in identifying genes expressed within the SCN at several points in development. Among them is the highly conserved TF, Six3. Interestingly, Six3 was shown expressed within the early SCN neuroepithelium and continued to be expressed in all SCN neurons

through adult stages, where expression within the nuclei was found to cycle between periods of light and dark (VanDunk et al., 2011).

Six3 is known to act at several stages in cellular development. Through interactions with Wnt signaling pathways, Pax6, and Geminin (Kobayashi et al., 2002; Lagutin et al., 2003; Gestri et al., 2005; Lavado et al., 2008), Six3 plays an essential role in patterning the mammalian forebrain. In addition, Six3 has been shown to be critical in cellular proliferation (Del Bene et al., 2004), specification of neuronal subtypes (Liu et al., 2010), regulation of gene expression (Manavathi et al., 2007), and lens formation (Liu et al., 2006) during eye development. Recently, Six3 was additionally found necessary for the formation of the SCN (VanDunk et al., 2011). We demonstrated that deletion of Six3 specifically from neural progenitors not only eliminated the presence of dense cell clusters along the ventral anterior forebrain, but also the expression of several SCN-genes including the TFs Lhx1 and Rora, and the peptide arginine vasopressin (AVP).

The SCN are not the only nuclei with Six3 expression into adult stages. Cell types within the retina and several nuclei derived from the telencephalon and diencephalon also retain Six3 expression (Conte et al., 2005). The continued expression of Six3 within the SCN and other brain regions as well as its known roles within eye development suggests this gene may be playing additional roles postmitotically. Herein, we examine the effects of deletion of Six3 postmitotically in mice with emphasis on the SCN, by targeting deletion from GABA-ergic populations, as most, if not all, neurons of the SCN express GABA (Okamura et al., 1990). Specifically, we investigated the role of postmitotic

expression of Six3 in the specification or maintenance of SCN neuronal subtypes, with focus on neurons expressing TFs *Rora*, *Lhx1*, *Per1* or the neuropeptides AVP or vasoactive intestinal polypeptide (VIP). We find that Six3 is not necessary for proper expression or localization of postnatal TF or peptide mRNA. However, we demonstrate that postmitotic expression of Six3 has a profound impact on animal growth, suggesting a role for Six3 in nuclei regulating growth hormones, appetite, or motivation.

**Materials and Methods**

*Animals and housing.* Male and female wild-type C57BL/6 (Charles River), *Six3<sup>fl</sup>ox* (Liu et al., 2006) (Guillermo Oliver, St. Jude Children's Research Hospital, Memphis, TN), *Gad2-cre* (010802; (Taniguchi et al., 2011) The Jackson Laboratory), and *Rosa26-tdTomato* (007909; (Madisen et al., 2010), The Jackson Laboratory) were used. Mice were maintained in a normal 12 h light/dark schedule under standard care conditions. Mutant animals were maintained on a C57BL/6 background (Charles River). All experiments were approved by the Animal Studies Committee at Washington University and followed NIH guidelines.

*Genotyping.* Mice were genotyped by PCR for *Gad2-cre*, *Six3* floxed, and/or wild-type alleles as described previously (Liu et al., 2006) or as outlined by The Jackson Laboratory. The presence of Cre was determined by the presence or absence of an amplified band as previously described (VanDunk et al., 2011).

*Tissue acquisition.* Unless otherwise stated, all collections were done at approximately zeitgeber time (ZT) 8. Neonatal pups [postnatal day 0 (P0) to P2] or embryos [embryonic day 10.5 (E10.5) to E18.5] from timed pregnant females (morning of plug was E0.5) were anesthetized on ice and either transcardially perfused ( $\geq$ E16.5) or immersion fixed in 4% paraformaldehyde (PFA) in 0.1 M PBS, pH 7.4. Older mice were anesthetized with a ketamine/xylazine mixture before perfusion with 0.9% saline followed by 4% PFA. All

tissues were post fixed in 4% PFA overnight at 4°C, cryoprotected in 20% sucrose in PBS, frozen in O.C.T. Compound Embedding Medium (Tissue-Tek), and stored at -75°C. Serial sections (20µm) were cut on a Hacker cryostat and thaw mounted on Superfrost Plus slides (Thermo Fisher Scientific). Five adjacent sets of sections were prepared from each postnatal age and stored at -20°C.

*Probe synthesis.* Plasmids for *in situ* hybridization probes were acquired as previously described (VanDunk et al., 2011). Gene fragments from verified plasmids were linearized by direct amplification by sequence or vector-specific PCR. Digoxigenin (DIG)-labeled anti-sense and sense RNA probes were made using PCR products as template and T7, T3, or SP6 RNA polymerases (Roche). cRNA probes were purified using Quick Spin columns (Roche) and quantified by spectrophotometry. Probes were used at a concentration of 1-2 µg/ml. Sense counterparts of all probes were tested to ensure probe specificity.

*In situ hybridization.* Slides were immersed in 4% PFA, permeabilized with proteinase K, returned to 4% PFA before being washed in 0.1 M triethanolamine-HCl with 0.25% acetic anhydride. Once blocked in hybridization buffer at 65°C slides were incubated in hybridization buffer containing 1-2 µg/ml DIG-labeled anti-sense cRNA overnight at 65°C. Slides were then washed in 2XSSC buffer at 62°C, washed in 0.2XSSC at 65°C, blocked with 10% normal horse serum (NHS) in 0.1M PBS, and incubated in alkaline

phosphatase labeled anti-DIG antibody (1:2000 in 10% NHS; Roche) overnight. Sections were washed and color was visualized using Nitro blue tetrazolium and 5-Bromo-4-chloro-3-indolyl phosphate (Roche). Staining was stopped after visual inspection. Sections were washed, fixed in 4% PFA, and coverslipped in 90% glycerol, Vectashield Mounting Medium (Vector Laboratories), or UltraCruz Mounting Media with DAPI (Santa Cruz Biotechnology).

*Image acquisition.* Images were acquired using a Nikon Eclipse 90i microscope, Photometrics Coolsnap HQ2 camera with a Prior Scientific ProScan II motorized translation stage, and acquired in Volocity (PerkinElmer Life and Analytical Sciences). Images were exported as 8bit JPEG or TIFF files. All images were adjusted for clarity by filtering and/or modifying levels, as necessary, in Photoshop (Adobe Systems).

*Analysis.* General SCN morphology was assessed by obtaining outlines drawn based on cellular density marked with nuclear DAPI stain and measuring individual nuclei for mid-SCN width, area, and cell density. Rostral to caudal length of the nuclei was calculated by counting the number of sections containing Rora staining and estimating length based on section thickness. Growth was measured by measuring weight every two to three days.



## Results

### Onset of Gad2-cre expression in the SCN

The onset of Gad2-cre recombination in the early SCN was assessed by crossing Gad2-cre expressing mice with reporter mice containing the Rosa26 locus with loxP flanked STOP cassettes followed by red fluorescent protein (tdTomato). In this manner, tdTomato was expressed following cre-mediated recombination in mice containing both transgenes. We found that while cre recombinase was present within the early SCN by E12.5, as marked by the overlap of Six3 and Lhx1, cre-mediated expression of tdTomato was not present until E13.5 (Figure 1). These data suggest successful recombination and consequent reporter protein expression events require approximately two days after cells of the SCN become postmitotic to be visualized.

### Gad2-cre driven conditional knock-out of Six3 leads to growth deficiencies

Crossing *Six3<sup>lox/+</sup>/Gad2-cre(+)* males x *Six3<sup>lox/lox</sup>* females yielded homozygous (*Six3<sup>lox/lox</sup>/Gad2-cre(+)*) and heterozygous (*Six3<sup>lox/+</sup>/Gad2-cre(+)*) mutants as well as homozygous and heterozygous *Six3 Gad2-cre(-)* controls in normal Mendelian numbers. We found P2 homozygous and heterozygous mutants and control littermates were similar in weight and disposition. However, *Six3<sup>lox/lox</sup>/Gad2-cre(+)* homozygous mutants did not undergo normal weight gain and growth as they developed, evident by P10 (Figure 2), which led to lethality around P21. Prior to death these animals displayed mild ataxic gate

and appeared hypoactive. No growth or disposition deficits were observed in *Six3<sup>fllox/+</sup>* *Gad2-cre(+)* heterozygous mutant animals.

### **Role of Six3 in postmitotic specification of SCN neurons**

To test whether Six3 was necessary for the postmitotic specification of SCN neurons and neuronal subtypes we analyzed *Six3<sup>fllox/fllox</sup>/Gad2-cre(+)* and *Six3<sup>fllox</sup>/Gad2-cre(-)* littermate controls for expression of TF and peptide mRNA and general SCN anatomy. Animals were observed at postnatal days 2 (P2, n=2) and 21 (P21, n=2). We found that Six3 was successfully eliminated from the SCN of P2 experimental mice while expression remained present in regions not expressing GABA, such as the pituitary gland (Figure 3).

Deletion of Six3 from postmitotic SCN neurons did not have any adverse effects on the general morphology of neural tissues in animals at P2 (data not shown). Through assessment of nuclear DAPI stain, indicating cellular density, we found the relative rostrocaudal positioning, size, and morphology of SCN nuclei remained intact following postmitotic Six3 deletion (Table 1). *In situ* hybridization for the expression of SCN TFs revealed that the whole SCN expression of Rora was maintained in these animals, as well as the expression and centralized localization of Lhx1. In addition, we found that the expression of AVP mRNA was maintained within the SCN shell (Figure 3).

Analysis of *Six3<sup>fllox/fllox</sup>/Gad2-cre(+)* P21 mice showed that although these animals displayed severe growth deficits, no gross morphological defects were present within the

neural tissue (data not shown). Further, general morphology and size of the SCN were maintained (Table 1). We found *Six3* was almost completely eliminated in *Six3<sup>flox/flox</sup>/Gad2-cre(+)* homozygous mutants (Figure 4). Remaining low levels may indicate either background expression, inefficiency in recombination, or expression of *Six3* in non-GABAergic cells. In the absence of *Six3* we found that mRNA expression levels and localization of the TFs *Rora* and *Per1*, as well as the peptide VIP, were maintained. In addition, the localization of *Rora* to the SCN shell, characteristic of mature SCN anatomy (VanDunk et al., 2011), and VIP to the core SCN remained. Together, these data suggest postmitotic *Six3* is not necessary for the specification of peptidergic neurons within the SCN or their anatomical localization. In addition, the presence of *Six3* postmitotically does not appear to influence TF expression or maturational changes in localization.

### **Gad2-cre conditional deletion of *Six3* may affect extra-SCN structures**

By analyzing the full rostral to caudal expression of *Gad1* and *Six3* mRNA within P2 neural tissue we found that co-expression was not unique to the SCN. At least six additional regions were found to contain strong overlap (Figure 5). These regions include: Amacrine cells of the inner nuclear layer of the retina, reticular hypothalamic nuclei, lateral arcuate nuclei, posterior pretectal/ olivary pretectal nuclei, substantia nigra pars reticulata (SNpr), and the medial terminal nucleus of the accessory optic tract (a full list of regions of potential overlap can be found in Supplemental Table 1). We find that

*Six3<sup>flox/flox</sup>/Gad2-cre(+)* mice successfully lose expression of Six3 in each of these areas (Figure 5B).

**Discussion:**

We have previously demonstrated that premitotic Six3 plays a critical role in the formation and specification of cells that will become the SCN (VanDunk et al., 2011). In the current study we found that Six3 is not necessary to shape SCN cell identity postmitotically. We found the presence and localization of SCN-expressed TFs Rora, Lhx1, and Per1 were maintained in Six3 knock-outs sampled at neonatal (P2) and functionally mature stages (P21). In addition, Six3 expression was not necessary for the onset or the localization of cells expressing AVP or VIP mRNA. These findings suggest development of TF and peptidergic subtypes within the SCN occur independent of postmitotic Six3 or, alternatively, that these regions are specified premitotically and are unaffected by the postmitotic loss of Six3. Successful recombination using Gad2-cre was first evident by E13.5, approximately two days after neurons of the SCN became postmitotic. This delayed recombination indicates SCN neurons may have already begun expressing postmitotic markers, such as Lhx1, before Six3 is functionally removed. Lhx1 is one of the first TFs expressed by postmitotic SCN neurons (VanDunk et al., 2011) and its early deletion has been suggested to influence peptidergic specification (Blackshaw et al., 2010). Therefore, it may not be surprising that Six3 loss after the onset of Lhx1 would not affect peptide expression in the SCN.

Although expression and localization of established TFs and peptides persisted following the postmitotic deletion of Six3, it is unknown whether the expression of Six3 during this period may aid in maintenance of clock gene rhythms or greater circadian

function. The severe growth impairment and early lethality seen in *Six3<sup>flox/flox</sup>/Gad2-cre(+)* experimental mice make it impossible to look for effects on circadian function using the standard assessment of locomotor rhythms through wheel-running behavior. However, other measures such as recording neuronal rhythmicity in the intact slice or dispersed cultures, which utilize tissues from younger animals, may be useful options. Alternatively, previous reports have shown that reductions in functional Six3 protein, by single-allele deletions, can lead to the failed activation of downstream targets such as Sonic hedgehog and Nkx2.1 (Geng et al., 2008). This suggests that single-allele deletions may be enough to show changes in normal Six3 function. While *Six3<sup>flox/+</sup>/Gad2-cre(+)* heterozygous mutant animals did not display abnormal growth patterns, these animals could be used as an alternative way to assess the effects of postmitotic Six3 deletion on circadian function.

The severe runting phenotype displayed by Six3 postmitotic knock-outs was surprising given the normal development of the pituitary gland, the main producer of hormones regulating growth and development (Sam and Frohman, 2008). It is likely then that the deletions of Six3 from extra-SCN regions showing co-localization with Gad1 are contributing to the failure of normal growth in these animals. The progressive nature of this phenotype closely resembles the effects seen in animals with highly reduced levels of dopamine. Szczypka and colleagues (1999) demonstrated that animals, lacking normal levels of circulating dopamine become progressively hypoactive and hypophagic causing lethality around the time of weaning (Szczypka et al., 1999). Both the arcuate nucleus

and the substantia nigra; areas of dopamine production in the brain, show overlap in the expression of *Six3* and *Gad1* mRNA. While not producing or secreting dopamine itself, the SNpr, is thought to regulate dopaminergic activity within the pars compacta (Deniau et al., 1982). We hypothesize that the deletion of *Six3* from the arcuate and/or SNpr plays a role in the ability of these nuclei to produce functional levels of dopamine for maintaining aspects of appetite and motivation (Salamone and Correa, 2009; Fulton, 2010; Kurniawan et al., 2011). It is known that in addition to the production of dopamine, the arcuate nuclei are responsible for secreting growth hormone releasing hormone, neuropeptide Y, and agouti-related proteins that act on hypothalamic nuclei and aid in regulation of growth and appetite (Broberger et al., 1998; Schwartz et al., 2000; Williams et al., 2001; Minor et al., 2009). The role of *Six3* deletion on these critical functions of the arcuate could shed light on the resulting growth deficiencies seen in our animals.

Previous studies have implicated the TF *Six3* in several aspects of cellular development including promoting proliferation (Kroll, 2007), differentiation (Kroll, 2007), and specification (Lagutin et al., 2001; Zhu et al., 2002; Ohyama et al., 2005). Here we reveal that *Six3* does not play a role in the specification of at least several TF or peptidergic phenotypes in postmitotic SCN neurons. However, it is likely that *Six3* is playing a role in either the specification or function of nuclei necessary for growth, which may include areas responsible for regulation of growth signals, appetite, or motivation. Future studies will need to determine the key genes necessary for regulation of the distinct stages of cellular development of SCN neurons. Better understanding of the

functionality of these genes will advance our knowledge of how the SCN and its subdivisions are developmentally specialized to create and maintain this unique time keeping system.



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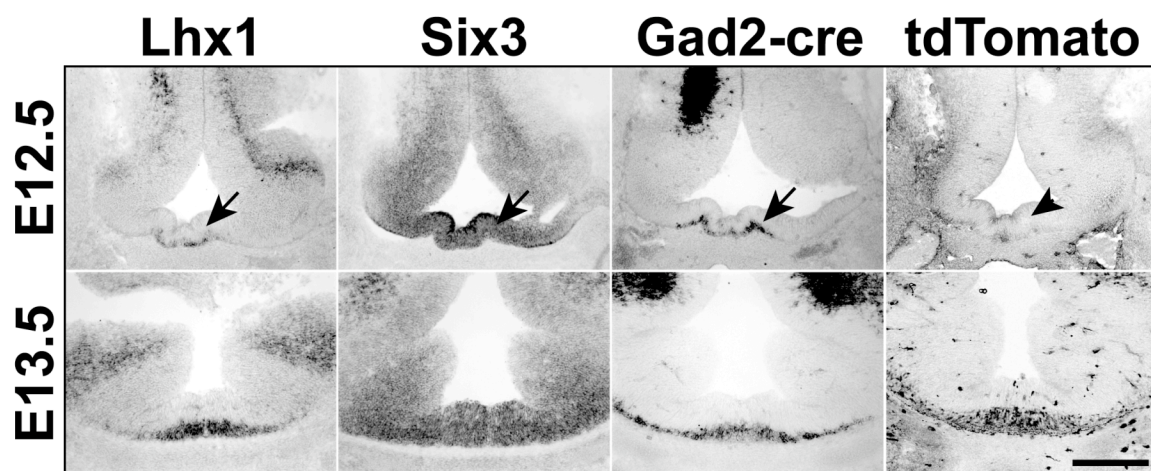
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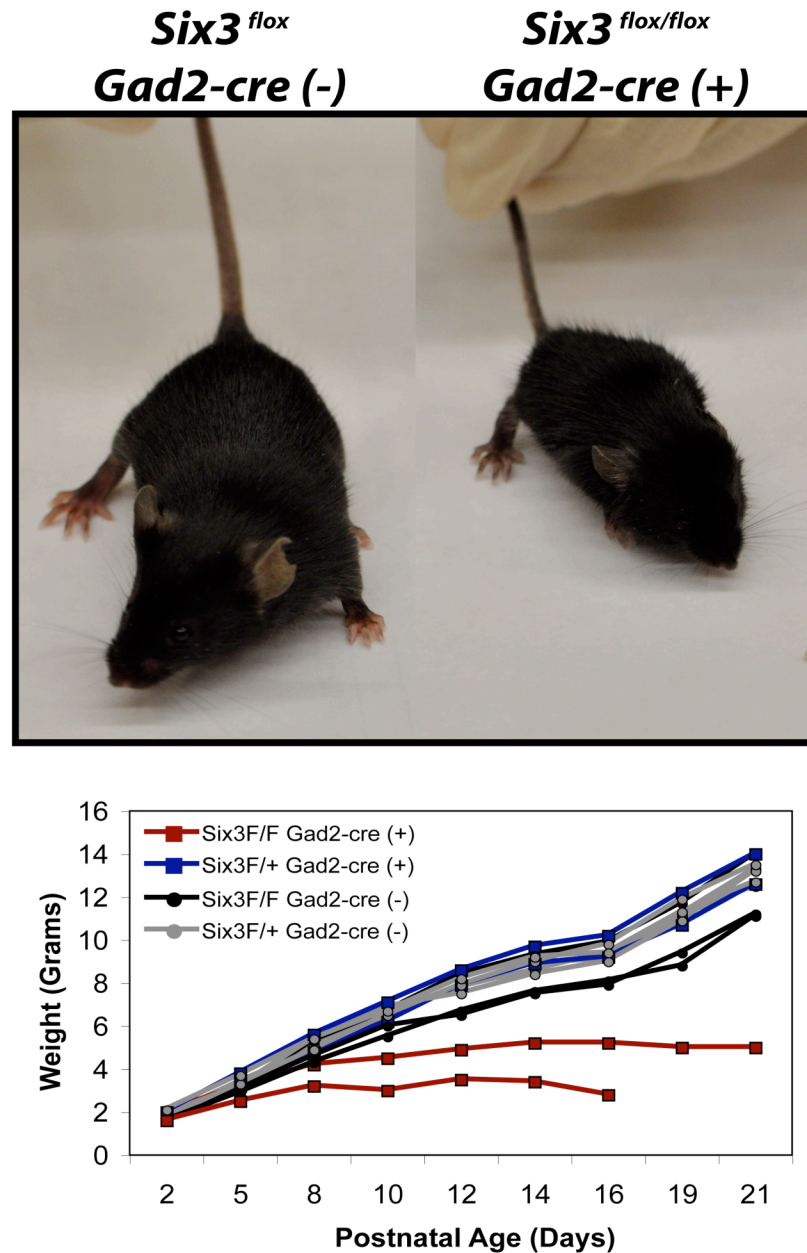
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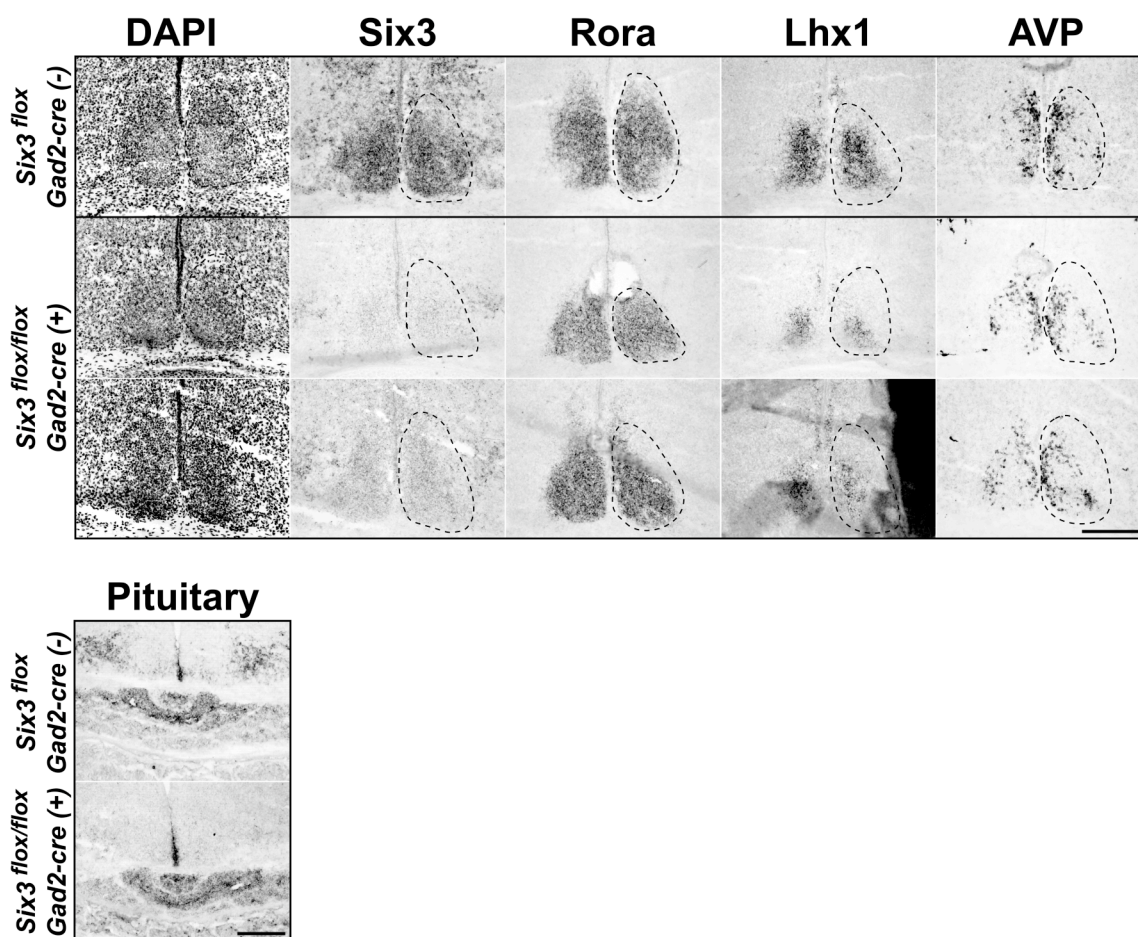
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**Figure 1.** Gad2-cre induces successful recombination within the SCN. Representative ISH images in adjacent transverse sections through the developing SCN show expression of Lhx1, Six3, Gad2-cre, and intrinsic tdTomato at E12.5 and E13.5. Image depicts the overlap of expression of Gad2-cre recombinase and Six3 within the early SCN, marked by the expression of Lhx1 (arrows in E12.5 tissue), and the successful recombination leading to tdTomato expression present by E13.5. Arrowhead points to absence of tdTomato signal at E12.5. Scale bar, 250  $\mu$ m.

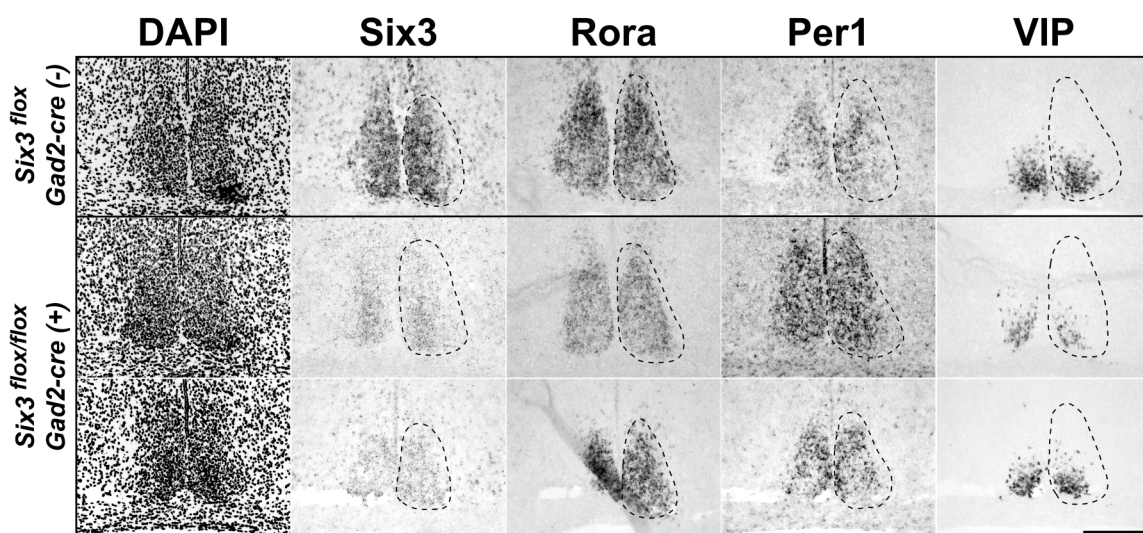


**Figure 2.** *Six3<sup>flox/flox</sup>/Gad2-cre (+)* mice display severe growth deficiencies visible by P10. *Top*, photograph of control *Six3<sup>flox</sup>/Gad2-cre(-)* mouse (left) shown beside a *Six3<sup>flox/flox</sup>/Gad2-cre(+)* experimental littermate (right) imaged at P23. *Bottom*, bar graph traces the weights (g) of 12 pups from a cross between *Six3<sup>flox/+</sup>/Gad2-cre(+)* male x *Six3<sup>flox/flox</sup>* female showing that experimental pups are born at a normal weight but stop increasing in size approximately eight days after birth.



**Figure 3.** Neonatal localization of SCN transcription factors and peptide expression persist in *Six3<sup>flox/flox</sup>/Gad2-cre(+)* mice. Inverted nuclear DAPI staining and ISH images show cell density and expression of Six3, Rora, Lhx1, and AVP in two P2 *Six3<sup>flox/flox</sup>/Gad2-cre(+)* mice compared with a *Six3<sup>flox</sup>/Gad2-cre(-)* littermate control. Postmitotic Six3 knock-outs display complete Six3 loss in GABA-ergic cells of the SCN while sparing Six3 expression within regions not expressing GABA, such as the pituitary gland. Expression of Rora, Lhx1, and AVP remain present and appropriately localized in *Six3<sup>flox/flox</sup>/Gad2-cre(+)* experimental animals. Dotted lines indicate margins of the SCN estimated from DAPI stain. Scale bar, 250  $\mu$ m.



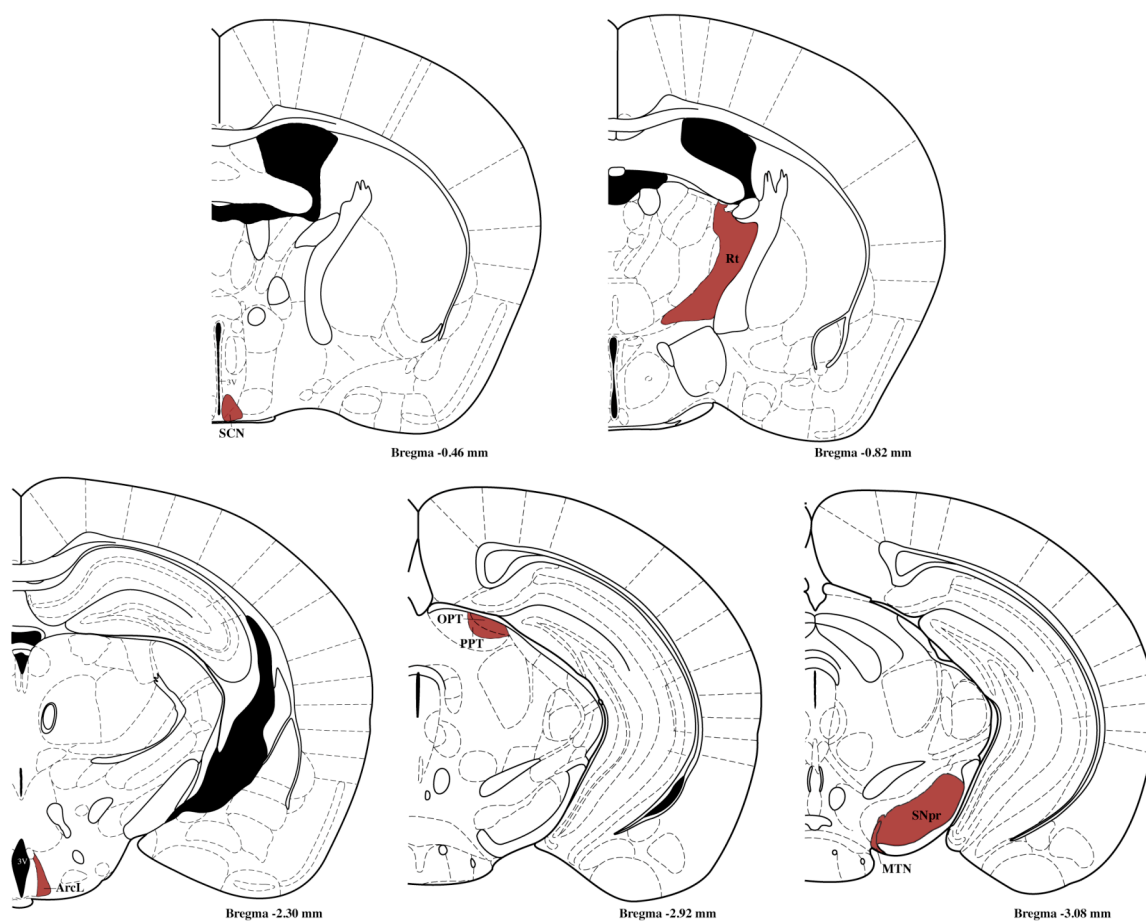


**Figure 4.** Postmitotic loss of *Six3* does not affect mature transcription factor expression or peptide localization. Inverted nuclear DAPI staining and ISH images show cell density and expression of *Six3*, *Rora*, *Per1*, and *VIP* in two P21 *Six3<sup>flox/flox</sup>/Gad2-cre(+)* mice compared with a *Six3<sup>flox</sup>/Gad2-cre(-)* littermate control. ISH for *Six3* mRNA reveals near complete loss in the SCN. Expression of *Rora*, *Per1*, and *VIP* remain present and localized in *Six3<sup>flox/flox</sup>/Gad2-cre(+)* experimental animals. Dotted lines indicate margins of the SCN estimated from the cell density marker DAPI. Scale bar, 250  $\mu$ m.

**Table 1.** SCN size is maintained in animals with postmitotic deletion of Six3.

Age, Genotype	Average mid-SCN Area (square microns)	Rostral to Caudal Length (um)	mid-SCN width (um)
<b>P2, Six3F Gad2-cre (-)</b>	106,114 ± 2,190	370 ± 50.00	311.63 ± 12.18
<b>P2, Six3F/F Gad2-cre (+)</b>	97,293 ± 4,773 $p=0.165$	370 ± 50.00	289.37 ± 12.46 $p=0.249$
<b>P21, Six3F Gad2-cre (-)</b>	99,759 ± 3,537	470 ± 50.00	274.21 ± 14.12
<b>P21, Six3F/F Gad2-cre (+)</b>	86,942 ± 13,664 $p=0.424$	420 ± 0.00	259.05 ± 17.23 $p=0.461$

Table displays mid-SCN area (calculated by averaging measurements of right and left nuclei for each animal), rostral to caudal length, and mid-SCN width of SCN nuclei ( $\pm$  s.e.m.), showing no overall changes in SCN size in P2 or P21 mice after postmitotic deletion of Six3.



**FIGURE 5A**

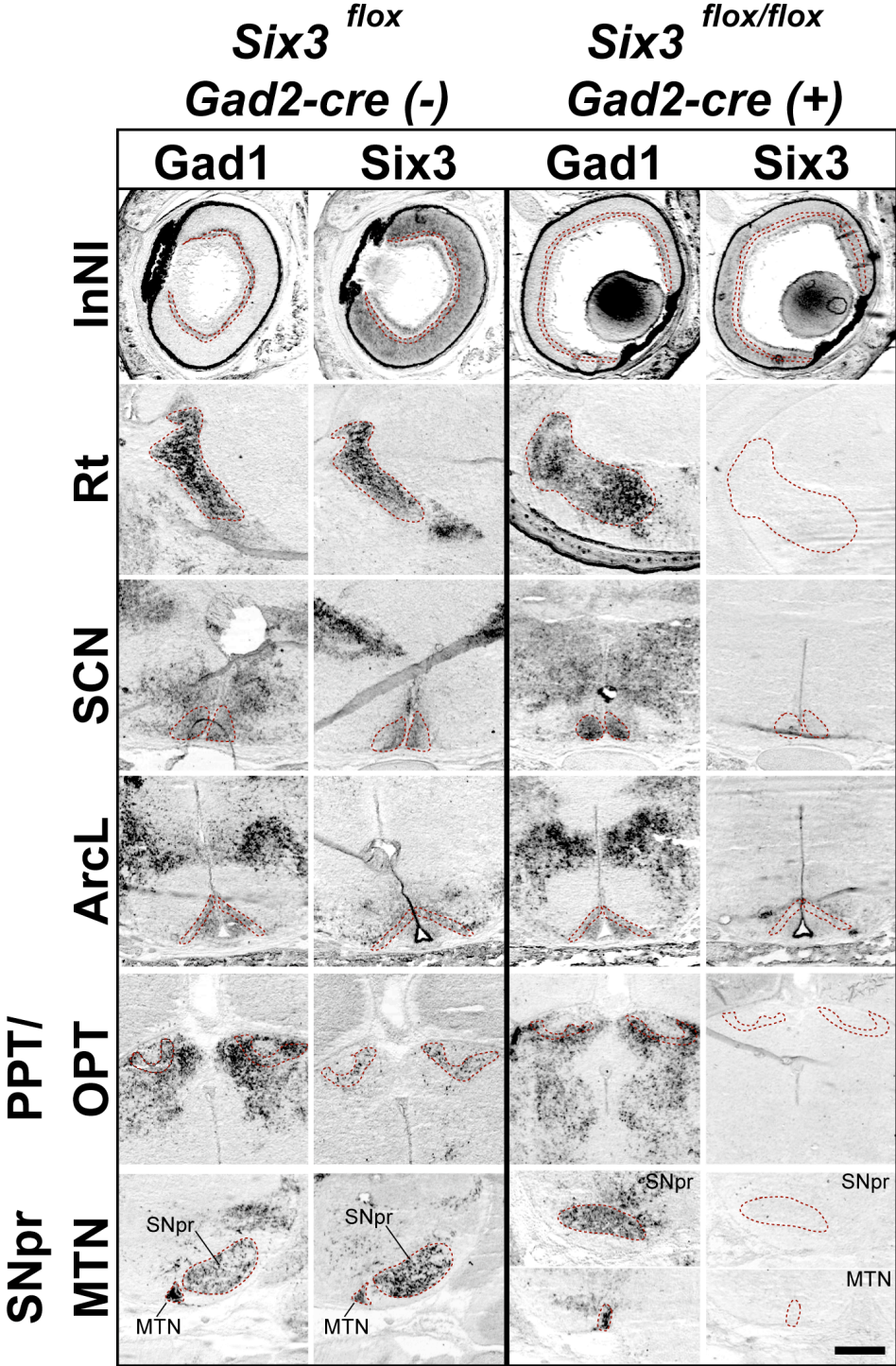


FIGURE 5B

**Figure 5.** Co-expression of Gad1 and Six3 mRNA is not exclusive to the SCN. Regions of most significant overlap between the expression of Gad1 and Six3 mRNA observed in P2 mice are displayed as red filled in regions within sections from an illustrated Adult mouse atlas (Paxinos and Franklin, 2001) in transverse section (**A**) and dotted red outlines in representative ISH images from adjacent transverse sections of control and *Six3<sup>flox/flox</sup>/Gad2-cre(+)* mutant mice (**B**). Figures depict significant overlap in amacrine cells of the inner nuclear layer of the retina (InNI), Suprachiasmatic nuclei (SCN), Reticular nuclei (Rt), lateral Arcuate nuclei (ArcL), Posterior pretectal nuclei (PPT)/Optic pretectal nuclei (OPT), Substantia nigra pars reticulata (SNpr) and medial terminal nucleus of the accessory optic tract (MTN). *Six3<sup>flox/flox</sup>/Gad2-cre(+)* homozygous mutants show successful deletion of Six3 from all regions of overlap. Scale bar, 500  $\mu$ m.

**Supplemental Table 1.** Regions of overlap of Six3 and Gad1 mRNA in P2 mouse neural tissue.

<b>Regions of potential overlap</b>
Amacrine cells of the inner nuclear layer, retina
Accumbens nucleus, core
Stria terminalis
Septohypothalamic nucleus
Reticular thalamic nucleus
Zona inserta
Suprachiasmatic nucleus
Anterior amygdala area basolateral
Paraventricular hypothalamic nucleus, medial parvicellular part
Interstitial nucleus of the posterior limb of the anterior commissure
medial preoptic nucleus, medial part
Aruate hypothalamic nucleus, lateral part
Posterior pretectal nucleus/Olivary pretectal nucleus
Posterior hypothalamic nucleus
Retroparafascicular nucleus
Interstitial nucleus of Cajal
Substantial nigra, pars reticulata
Nucleus of Darkschewitsch
Medial geniculate nucleus
Subbrachial nucleus

Table lists all the potential brain regions with overlapping expression of Six3 and Gad1 mRNA in order from rostral to caudal.

## **-CHAPTER 5-**

### **Conclusions and Future Directions**

The primary goal of this thesis was to identify genes expressed by SCN neurons outside of those involved in the molecular clock, and to use those genes to elucidate mechanisms necessary for specification and development of SCN neurons, the SCN network, and circadian function. The SCN are not only essential nuclei necessary for regulating homeostatic processes but represent highly specialized nuclei with a distinct function and thus can be utilized as a model for understanding the direct relationships between genes and rhythmic behavior.

Combinations of transcription factors (TFs), in part, specify the diverse number of cell types that reside in and generate the complexity of the brain; however the direct relationship between TFs and the SCN has never been explored. We pioneered this relationship by utilizing a pre-existing TF screen (Gray et al., 2004) to identify candidate TFs expressed relatively specifically within the prenatal and postnatal SCN (Chapter 2). These candidate TFs allowed us to not only characterize postnatal development of the SCN, outside of the consideration of clock genes but more importantly provide the first descriptions of prenatal maturation of SCN nuclei. In addition, these descriptions provided a tool with which we could investigate the contribution of other elements on development of SCN network organization by assessing the maintenance of mature TF patterns. Finally, we took the first steps into identifying TFs responsible for SCN development by demonstrating the necessity of *Six3* in both SCN formation and specification. Thus, this work has given the field of circadian biology a foundation for understanding the development of the mammalian principal circadian rhythm generators.



**Development of SCN neuronal subtypes**

A reoccurring theme in the field of circadian biology is how to reconcile the heterogeneity of SCN cellular subtypes (differences in afferent input, connectivity, neuropeptide content, etc.) with possible regions of functional differences and the homogeneous nature of rhythmic capacity within SCN neurons. The presence of cellular heterogeneity has prompted the division of the SCN into anatomically and functionally discrete zones. Classification using different subtypes yields different, not always opposing, ways in which to classify regions of the SCN. Our data demonstrate that the SCN can be subdivided in yet another way based on adult patterns of TF expression that partially overlap with classic peptidergic divisions. Specifically, we find mature localization in expression of *Lhx1* in a central region of the SCN partially overlapping regions expressing both AVP, as well as VIP. We also find the expression of *Rora* is mainly limited to regions containing AVP expressing cells. However, both *Rora* and *Lhx1* expression can be seen in cells that do not express either VIP or AVP. This additional method of dividing SCN neurons into subtypes reiterates the claims argued by Morin, 2007 that broad anatomical classification of SCN subdivisions are an oversimplification and that the SCN reflects subtypes much more diverse (Morin, 2007). Even still, understanding the relationships of these subtypes with one another may lead to a more thorough view of SCN network function.

Early studies performed by Altman and Bayer (1986) describe SCN neurons being derived from two distinct neuroepithelial zones, giving rise to the hypothesis that

SCN precursor cells may exist with distinctly restricted fates even before cells become postmitotic. In this work, we developed tools that allowed us to test the hypothesis that SCN precursors exhibit different genetic markers prior to SCN formation. Our results do not support a genetic distinction in the neuroepithelium from which SCN neurons are derived. On the contrary, we find the entire SCN neuroepithelium expresses a combination of *Six3*, *Six6*, and *Fzd5*. Additionally, SCN neurons progress through the same gene cascade from the time of precursors to being postmitotic. From the proliferative neuroepithelium cells undergo mitosis and lose expression of *Fzd5* and begin expressing *Dlx2*. Once cells become postmitotic they transiently express *Dlx2* followed by *Lhx1*. The first sign of variability between cells is in the onset of peptide expression and the loss of *Lhx1* expression in cells located in the outer SCN, leading to the visual appearance of centralization of *Lhx1*, present by E18.5. Thus, the genetic markers developed support the idea that SCN neurons are derived from the same precursor population.

Several studies indicate that cell-to-cell signaling in the SCN promotes rhythmicity in a subset of SCN neurons (Yamaguchi et al., 2003; Aton et al., 2005; Maywood et al., 2007). Thus, although each cell has the capacity to create rhythms, some produce autonomous rhythms, while others can be driven to rhythmicity through network function. Webb and colleagues (2009) have shown that neurons displaying cell-autonomous rhythms can express VIP, AVP, or neither peptide, suggesting no one peptidergic subtype of SCN neuron can be attributed to those displaying cell autonomous

rhythm-generating properties. They also demonstrate that single cells have the ability to shift between rhythmic modalities.

Taken together these results give rise to the hypothesis that all SCN cells are innately similar. SCN precursors follow the same gene cascade that specifies them to be “SCN” neurons and intrinsic oscillators. As they develop, these unstable oscillators undergo further specialization in order to become a functional network. Differentiation of these neural precursors into unique subtypes may then arise from one or several sources such as morphogens or signaling elements, connectivity, environmental influences, innervation, or other genes yet to be discovered. Interestingly, it has been suggested that variability in capabilities of single oscillators to maintain similar amplitude and periodicities from cell to cell aids in robustness and plasticity of the circadian clock network (Webb et al., 2009). We further hypothesize that diversity in neurotransmitter phenotype, response to stimuli, or TF expression of individual oscillators may also contribute to the plasticity of the network.

### **Differential gene expression and the impact of retinal innervation on SCN development**

Several researchers have found differential expression of clock genes across the SCN at mid-day time points, namely in the expression of *Per1* and *Per2* isolated to the SCN shell. In Chapter 2 we find that the mature SCN displays patterns of *Rora* expression similar to that described for *Per1* and *Per2*. This pattern of expression is

progressively localized through development. Interestingly, expression of another gene in this study, *Lhx1*, moves centrally during prenatal development. As of yet, the meaning behind the localization of these TFs is unknown, but it is tempting to speculate that the localization could form a functional division in the SCN. The progressive regionalization of *Rora* is coincident with several anatomical and functional changes within the nuclei including retinal innervation and synaptogenesis. We hypothesize that the progressive change in postnatal (and prenatal) TF localization may represent functional or induced changes within the nuclei.

In Chapter 3 we investigated the role of two crucial aspects of the circadian system in shaping SCN development. We find that the circadian pacemaker develops anatomically independent of retinal innervation or VIP/VPAC<sub>2</sub> signaling. Specifically, when VIP, VPAC<sub>2</sub>, or retinal innervation are eliminated we found that subdivisions, in way of peptide segregation and TF localization, are formed and SCN cell number and nuclei size persist.

Despite the lack of gross anatomical changes, effects on circadian period have been repeatedly reported in animals lacking retinal innervation. Effects of circadian period lengthening have been observed in mice with both genetic (Wee et al., 2002) and naturally occurring models (Laemle and Ottenweller, 1998) of enucleation. In addition, it has been shown that altering the photoperiod of neonatal mice can impact the developed circadian period (Ciarleglio et al., 2011). These findings suggest that although not necessary for development of the anatomical timekeeping system that retinal innervation

and/or light input may be necessary to initially set the timing of the circadian period by an unknown mechanism. These results are consistent with there being a baseline innate system of individual clocks that would then need to be structured on a molecular level to set the timing of the network.

### **Role for Six3 in development**

In Chapter 2 we identified the homeobox gene, Six3, as critical for formation and early specification of the SCN. The premitotic loss of Six3 led to loss of cell dense clusters along the ventral surface of the developing telencephalon/diencephalon indicating loss of nuclei formation. In addition, loss of Six3 led to the lack of cells expressing key downstream genes such as Lhx1 and Rora. This represents the first gene to be discovered as playing an early critical role in development of the SCN.

Six3 expression is maintained in several nuclei in mature animals. The prolonged expression of Six3; a gene predominately thought of as involved in early patterning, led to the possibility of additional roles for this gene in postmitotic development and function. In order to target deletion from the SCN postmitotically, we generated mice with deletion of Six3 driven by Gad2, a precursor to GABA, expressed by SCN neurons (Okamura et al., 1990). We find that Gad2 recombination is apparent by approximately two days after cells exit the cell cycle. In Chapter 4 we demonstrate that postmitotic loss of Six3 had gross effects on the development of mice containing homozygous mutations, leading to severe growth deficiencies and early lethality. This indicated two things; first,

Six3 plays an active role in cells postmitotically and second, that Six3 may have a strong role in postmitotic regulation of nuclei responsible for coordinating growth. We find postmitotic deletion of Six3 within the SCN had no effect on the subsequent expression and localization of TFs and peptides; however a role in function has not yet been investigated.

## **Future Directions**

### **SCN formation and specification**

Early specification relies not only on locally expressed TFs but also on extrinsic morphogens and other signaling peptides. Through our characterizations we found Lhx1 and Six3 could mark the early developing SCN. We then used these markers to identify, for the first time, where the immature SCN is located within the complex early signaling environment of the developing forebrain. Hedgehog, Wnt, bone morphogenetic protein (BMP), and fibroblast growth factor (FGF) signaling have all been implicated in shaping aspects of early telencephalon/diencephalon development (Hogan, 1999). In Chapter 2 we looked for the expression of a subset of signaling receptors and cues within the SCN. We found the SCN neuroepithelium expressed Fzd5, a receptor for Wnt signaling, but did not express SHH, the SHH receptor, Patched1, or BMP7. Future work should seek to further identify the signaling molecules and gradients that aid in the specification of SCN neurons. Understanding if different dorsal to ventral regions of the early SCN

neuroepithelium are responding differentially to signaling morphogens would shed light on the how SCN neuronal subtypes might be formed.

### **Six3 and the SCN**

While we were able to demonstrate the essential involvement of Six3 in the early development of the SCN, many questions remain. Future work should focus on investigating (1) how Six3 regulates initial SCN specification (2) possible mechanisms for Six3 in SCN functionality and (3) use of Six3 for genetic ablation of the SCN.

Six3 has been shown to interact with a number of genes to direct early forebrain patterning, cell proliferation, specification, and differentiation (Del Bene et al., 2004; Liu et al., 2006; Manavathi et al., 2007; Liu et al., 2010). However, the possible relationship between these genes, such as Geminin (Kroll, 2007) and Pax6 (Liu et al., 2006) and the SCN is unknown. In order to understand how Six3 directs formation of the SCN, further experiments focusing on the presence of Six3 target genes within the developing SCN need to be conducted. Assessing genes elevated or reduced in the absence of Six3, through a DNA microarray, could aid in selection of downstream target candidates.

In Chapter 4 we assessed the role of Six3 in postmitotic development of the SCN. Investigation in this manner addressed the role for Six3 in postmitotic cellular/ network development as well as maintenance of nuclei function. We found postmitotic deletion of Six3 did not affect the specification of SCN TF-expressing or peptidergic neuronal subtypes; however assessment of contribution into functional elements could not be

assessed by traditional methods due to early lethality. Multi-electrode array recordings, as well as luciferase conjugated constructs have proven to be valuable resources to assess SCN network function and connectivity in addition to the rhythmicity of individual cells (Welsh et al., 1995; Liu et al., 2007). These methods normally utilize tissue taken from animals within the first ten postnatal days, thus these alternative methods could be very useful in identifying any changes in SCN network function or rhythmic properties of single cells using the *Six3<sup>lox/lox</sup>/Gad2-cre(+)* mice we have generated. Alternatively, the runting phenotype could be alleviated by conditional deletion of *Six3* after a mature network has been formed. This could be achieved through use of mice with an inducible form of cre recombinase (ie. tamoxifen) driven by a promoter expressed by SCN neurons (such as the existing *Six3CreER<sup>T2</sup>* (Geng et al., 2008)). The inducing agent could be administered to the entire animal (IP injection or oral gavage) or stereotactically injected into the SCN for targeted *Six3* deletion. Animals with postnatal deletion of *Six3* could then be tested for SCN function through assessment of wheel running activity within multiple lighting paradigms.

The severe growth deficits seen within postmitotic *Six3* homozygous mutants provides evidence that *Six3* is playing critical roles in either the postmitotic specification or regulation of cells/nuclei necessary for growth. Assessment of regions with overlap of *Six3* and *Gad1* mRNA expression allow us to pinpoint potential nuclei responsible for these changes. However, given that *Six3* remains transient for a number of populations (Conte et al., 2005; VanDunk et al., 2011), assessment at P2 may not be sufficient for



determining the breadth or number of nuclei affected. Assessment at earlier stages in development may provide a more accurate understanding of the type of nuclei that may be affected by the conditional deletion. Alternatively, whole brain or isolated tissue lysates could be tested for changes in the overall levels of major neurohormones as deficits in growth and early lethality have been linked to a number of factors including reduced dopamine (Kobayashi et al., 2004) and PACAP (Gray et al., 2001; Sherwood et al., 2007). Identifying hormones affected would aid in detection of nuclei requiring postmitotic Six3.

Six3 is not exclusively expressed by the SCN neuroepithelium but rather is broadly expressed within the anterior forebrain early in development (Seo et al., 1999). In Chapter 2 we used a Nestin driven cre recombinase to remove Six3 expression from neural precursors. We found that Six3 deletion in this manner completely eliminated SCN formation and TF expression. In addition, we found no gross effects in neural morphology; however, postnatally these mice mimicked the phenotype of animals with postmitotic Six3 deletion and displayed severe growth deficiencies. It cannot be known whether this phenotype is due to effects on neuroepithelium of other forebrain nuclei influencing initial development or an effect of postmitotic control of nuclei regulating growth.

We find that the proliferative zone of the SCN neuroepithelium expresses Fzd5 and that cells of the SCN transiently express Dlx2 before becoming postmitotic. Using a Cre/loxP method with focus on these gene targets would aid in the isolation of Six3

deletion specifically from SCN precursors. Isolation of *Six3* deletion specifically from SCN neuroepithelium would allow for more precise investigation into the role of *Six3* in SCN development by eliminating the caveat of deletion from surrounding tissue. In addition, this specific deletion, if shown to alleviate effects on animal growth, could then be used as a model of genetic SCN ablation. A model of SCN ablation could be used to further studies on the impact of the SCN on development and rhythms within peripheral clocks as well as implications of loss of clock function on body temperature, sleep, and other homeostatic functions. Disruption of these key homeostatic mechanisms has been shown to lead to higher prevalence of both mental and physical disease (for review (Maywood et al., 2006; Bechtold et al., 2010)).

In conclusion, our efforts have created an invaluable list of TFs that serve as candidates for roles in SCN specification, development, and function. Even further we provide direct evidence that understanding the roles of TFs expressed within the SCN can reveal not only how these complex nuclei are formed and develop but also could provide critical tools for understanding the role of the SCN in animal behavior, homeostasis, and disease.

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Improved the technique of in-vitro fertilization and sperm cryopreservation and established embryonic stem cell lines from C57BL/6 mice within the Lab of Germline Modification.

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## **ABSTRACTS**

**2011 VanDunk C, Gray PA**

SCN Formation and Maturation: Influence of transcription factors, retinal innervation, and Vip signaling

Gordon Research Conference on Chronobiology, Lucca, Italy

**2010 VanDunk C, Gray PA**

Specific origin, dynamic maturation, and the necessity of Six3 in SCN development

Society for Neuroscience Conference, SanDiego, CA

**VanDunk C, Gray PA**

Transcription Factor Coding of the Mouse SCN

Sleep Research and Biological Rhythms Conference, Destin FL.

**2009 VanDunk C, Gray PA**

Ontogeny of the Mouse Suprachiasmatic Nucleus

Gordon Research Conference on Chronobiology, Newport, RI.

**2008 VanDunk C, Gray PA**

Characterization of gene expression profiles in the mouse suprachiasmatic nucleus Society for Neuroscience Conference, Washington D.C.

**2007 Gray PA, VanDunk C**

Developmental origins of ventral medullary NK1R neurons.

Society for Neuroscience Conference, SanDiego, CA

**2005 Nelson PG, Kuddo T, Song E, Dambrosia J, Kohler S, Satyanarayana G, VanDunk C, Grether J, Nelson KB.**

Measurement of trophins, peptides, and cytokines in blood of Down syndrome and autistic newborns and controls

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- 2004** Song EY, **VanDunk C**, Kuddo T, Nelson PG.  
Measurement of vasoactive intestinal peptide using a competitive fluorescent  
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**PROFESSIONAL AFFILIATIONS**

Society for Neuroscience